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The studies we have conducted under this contract have resulted in the mapping of the *continuous* antibody (Ab) binding epitopes on the protective H_C domain of BoNT/A with anti-BoNT/A antisera from several outbred species (including human). We have also mapped the *continuous* Ab and T cell epitopes that are recognized in two mouse strains (BALB/c and SJL) when each of BoNT/A or the H_C is used as an immunogen. Finally we determined the BoNT/A peptides that , when used as immunogens, give immune (Ab and/or T cell) responses that cross-react with H_C and/or with intact toxin. For BALB/c these are peptides 2, 3, 7, 10, 12, 17, 18, 21, 24 and 31; and for SJL they are peptides 4, 5, 6, 7, 8, 10, 15, 24 and 31. These studies have identified the BoNT/A synthetic peptides that represent potentially protective regions for incorporation into a synthetic vaccine.

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M. Zouhair Atassi 04-29-97

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ABBREVIATIONS

Abbreviations used in the text are:

Ab, antibody

BoNT, botulinum neurotoxin

BoNT/A, BoNT type A

BSA, bovine serum albumin

CFA, complete Freund's adjuvant

ConA, concanavalin A

$\mathbf{H_C}$, C-terminal fragment corresponding to residues 855–1296 of the heavy chain of BoNT/A

i.p., intraperitoneal

LNC, lymph node cells

MHC, major histocompatibility complex

PBS, 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2

RIA, radioimmune assay

s.c., subcutaneous

SD, standard deviation

S.I., stimulation index, which is: mean cpm incorporated *in vitro* by stimulated T cells/mean cpm incorporated by unstimulated T cells.

TeTX, tetanus toxin.

Key words: botulinum neurotoxin, synthetic peptides, antibodies, T-cells, epitopes

1. INTRODUCTION

Botulinum neurotoxin (BoNT) is a causative agent of food poisoning commonly known as botulism. The toxin is produced by *Clostridium botulinum* (Brooks, 1956), and is the most toxic substance known (Lamanna, 1959; Middlebrook, 1986; Sugiyama, 1980). It has been classified into seven distinct serotypes A through G (Hatheway, 1990). Most human toxicity is caused by serotypes A (60%), B(30%) and E (10%) and rarely F (MacDonald *et al.*, 1986; Tacket and Rogawski, 1989). *C. botulinum* is frequently used as a test organism in the food industry, while its neurotoxins are increasingly used in the study of nerve action as well as in the treatment of several neuromuscular disorders (Scott, 1989; Hoffman and Gartlan, 1993; Schantz and Johnson, 1993). A consequence of these applications is the required immunization of the personnel involved. For more than 30 years, formaldehyde-inactivated botulinum toxoid has been used to protect laboratory workers at high risk. However, the currently available pentavalent (ABCDE) toxoid does not display a consistent efficacy (Chan *et al.*, 1993; Middlebrook, 1995). It is unstable, gradually releasing active toxin and, therefore, is potentially hazardous and the production cost is rather high (Middlebrook, 1995). Immunological protection based on synthetic peptides should provide a stable alternative that would be expected to be safe and cost effective

The BoNTs are synthesized as single polypeptide chains (150 KDa). They are proteolytically processed (nicked) after secretion at about one-third the distance from their N-terminus to give an active form. The latter has two subunits comprising a light (L)-chain (50 KDa) linked by a single disulfide bond to a heavy (H)-chain (100 KDa) (DasGupta and Sugiyama, 1972; DasGupta, 1989). The usually fatal condition of botulism results from an irreversible blockade of neurotransmitter release from the nerve endings, causing neuromuscular paralysis. It has been possible to assign some functional activities to certain domains of BoNT. The C-terminal 50-kDa fragment of the H-chain, named H_C (Aguilera *et al.*, 1992), is implicated in the binding to the toxin receptor on the cell membrane (Kozaki *et al.*, 1989; Shone *et al.*, 1985). The N-terminus of the H-chain (H_N) is responsible for internalization (Shone *et al.*, 1987) of the L-chain into the nerve cells. The L-chain is an enzyme, recently identified as a zinc protease (Schiavo *et al.*, 1992), that blocks neurotransmitter release (Simpson, 1989). The complete amino acid sequences are now known for BoNT types A (Binz *et al.*, 1990; Thompson *et al.*, 1990), B (Whelan *et al.*, 1992), C1 (Hauser *et al.* 1990), D (Binz *et al.*, 1990), E (Poulet *et al.*, 1992; Whelan *et al.*, 1992), F (East *et al.*, 1992), and G (Campbell *et al.*, 1993). The primary structures of BoNTs show an extensive homology to that of tetanus toxin (TeTX) (Eisel *et al.*, 1986; Fairweather and Lyness, 1986), produced by *Clostridium tetani* (Niemann, 1991). These clostridial toxins share a number of structural and functional features (DasGupta, 1989; Matsuda, 1989; Simpson, 1989) in spite of radical differences in the clinical symptoms of poisoning. The sequence information facilitated the identification of residues involved in neurotoxicity on the L-chain (Kurazono *et al.*, 1992, Zhou *et al.*, 1995) as well

as a channel-forming motif on the H_N-fragment (Montal *et al.*, 1992) in BoNT type A (BoNT/A) and TeTX, thereby confirming the postulated function of each domain. Crystallization and preliminary X-ray analysis of BoNT/A have been reported (Stevens *et al.*, 1991).

It has been known that antibodies (Abs) to the receptor-binding regions on other bacterial toxins are very effective at neutralization. In fact, the H_C-fragment of TeTX was shown to provide protective immunity in mice against TeTX (Makoff *et al.*, 1989; Clare *et al.*, 1991). Recently, it was found (Clayton *et al.*, 1995; Middlebrook, 1995) that immunization of mice with H_C of BoNT/A afforded protection against a high challenge dose (10^5 LD_{50}) of the toxin, indicating that immunological mapping of this region of BoNT/A would be extremely valuable for a rational design of a synthetic peptide vaccine against BoNT/A. The overall goal of this research was to develop a synthetic vaccine against BoNT poisoning. The goal of the present phase was to determine, by a comprehensive synthetic strategy previously introduced by this laboratory (Kazim and Atassi, 1980, 1982), the *continuous* regions that are recognized by T- and/or B-cells (Abs) against H_C of BoNT/A (the terms *continuous* and *discontinuous* antigenic sites are defined in Atassi and Smith, 1978). The peptides that contain Ab and/or T cell epitopes will be used as immunogens in mice to identify those that stimulate immune responses which cross-react with H_C. In the second phase of the work (which was not funded), these peptides were then to be used in protection studies.

2. EXPERIMENTAL PROCEDURE

2.1. Synthetic Peptides, Toxoid and H_C

Thirty one overlapping peptides that started at residue 855 and encompassed the entire H_C domain of BoNT/A (residues 855–1296) (Fig. 1) were prepared by solid phase peptide synthesis on a benzyloxybenzyl alcohol resin (Peptides International, Kentucky) to which had been coupled 9-fluorenylmethylcarbonyl (Fmoc)-glycine (for peptides 1–30) or Fmoc-leucine (for peptide 31). The synthetic procedure employed Fmoc chemistry as described elsewhere (Atassi, *et al.*, 1991). After cleavage from the resin, the peptides were each washed three times with cold ether, dissolved in water, and freeze-dried. Peptides were desalted by gel filtration on Sephadex G-15 (Pharmacia, New Jersey) and purified on Vydac C18-reverse-phase HPLC columns using of 0.1% trifluoroacetic acid-acetonitrile gradients. Peptides 8, 9, 15, 27, 28 and 31 which contained Cysteine were purified under reduced conditions (the HPLC solvents contained 10% 2-mercaptoethanol). Purity of the synthetic peptides was checked analytical HPLC on a C18 column. The amino acid compositions of the peptides were also determined on acid hydrolysates of the peptides (0.1 mg in 0.25 ml of constant boiling HCl, 110°C for 24, 48, or 72 hrs). Tryptophan was determined by amino acid analysis of hydrolysates with p-toluenesulfonic acid containing 0.2% 3-(2 aminoethyl) indole (Liu and Chang, 1971). Analyses were done on a Beckman model 6300 amino acid analyzer. The values of serine and threonine were obtained by extrapolation to zero hydrolysis time. After purification,

the amino acid compositions of the synthetic peptides were in excellent agreement with those expected from their respective structures (Fig. 1).

Inactivated BoNT toxoid [absorbed, pentavalent (A, B, C, D, E)] was prepared by Bureau of Laboratories, Michigan Department of Public Health, Lancing, MI (Lot # 2). It was stored at 5° C, with thimerosal (0.01%) as a preservative. BoNT/A was purchased from Wako BioProducts (Virginia) as a solution in 0.05 M acetate-0.2 M NaCl, pH 6.0 and stored frozen (–20°C) until use. A completely synthetic gene encoding H_C of BoNT/A was constructed from oligonucleotides, expressed in *Escherichia coli,* and a full-size protein was produced (Clayton *et al.,* 1995).

2.2. Antisera

Horse antisera were prepared by s.c. immunization in multiple sites and every two weeks for over a year, with a formaldehyde-inactivated BoNT/A in Ribi adjuvant. The serum tested in the present studies was obtained after 4 injections. Human antisera were made against the pentavalent toxoid in human volunteers as described (Metzger and Lewis, 1979). The IgG fractions of these antisera were prepared and used in the binding assays. Non-immune horse sera obtained before immunization and normal human IgG were used as controls. Prior to the preparation of mouse antisera, the mice (outbred ICR from Harlan Sprague Dawley, Indiana; BALB/c and SJL from the National Cancer Institute, Frederick, MD) or the Jackson Laboratory, Bar Harbor, ME) were pre-bled to obtain their non-immune sera. The mice were immunized subcutaneously in the hind footpads with toxoid (ICR mice, 2.0 μg/mouse; BALB/c and SJL, 5.0 μg) or with H_C (0.25 or 0.5 μg). The antigen was given in a 50 μl emulsion of equal volumes of toxoid solution in PBS and CFA containing H37Ra strain of *Mycobacterium tuberculosis* (Difco, Michigan). The mice were given a booster every month for three months with a similar dose of toxoid, using incomplete Freund's adjuvant (Difco, Michigan) instead of CFA. Test bleeds were obtained 7–10 days after each injection and the final bleed was 10–14 days after the last booster. For each mouse strain, equal volumes of the preimmune sera or antisera of each bleed were pooled and kept at -20°C until used.

2.3. Radiolabeling of Protein A

Staphylococcal protein A (Pharmacia Biotech, Piscataway, NJ) was radiolabeled with ^{125}I (Amersham Corp., Arlington Heights, IL) using the chloramine-T method (Hunter and Greenwood,1962). Unbound ^{125}I was separated from the radiolabeled protein A by gel filtration on a column (0.8 x 20 cm) of Sephadex G-25 (Pharmacia Biotech, Piscataway, NJ), equilibrated with PBS containing 0.1% BSA (Sigma Chemicals, St. Louis, MO). At least 95% of the protein A-associated ^{125}I was precipitable with 10% (vol/vol) trichloroacetic acid.

2.4. Binding of Antibodies to BoNT/A or to the Peptides

Binding of anti-BoNT/A Abs to the synthetic peptides was determined by a solid–phase (plate) RIA. Flexible polyvinylchloride 96-well plates (Becton Dickinson, California) were coated (for 16 hr at room temperature) in triplicates with each of the 31 overlapping peptides (2.5 μg in 50 μl of PBS), H_C or toxoid (0.5 μg in 50 μl of PBS). Wells coated with proteins and synthetic peptides

unrelated to BoNTs were used as negative controls. The plates were washed five times with PBS and then blocked (1 hr, 37°C) with 100 µl of 0.5 % BSA in PBS. Prediluted horse antiserum (1:250, vol/vol with 0.1% BSA in PBS), human anti-BoNT/A immune IgG (1:1000 and 1:2000, vol/vol) or mouse antisera (1:500 and 1:1000, vol/vol) were added (50 µl) to the wells. After incubation for 20 hr at 4°C (or at 37°C for 3 hr), the wells were washed five times with PBS and then reacted (2 hr, 37°C) with 50 µl of affinity purified rabbit Abs against horse IgG (Cappel, North Carolina), or against human IgG (H + L) or against mouse IgG + IgM (Accurate Scientific, New York), prediluted (1:2000, vol/vol, with 0.1 % BSA in PBS). After washing five times with PBS, ^{125}I-labeled protein A was added to the wells (2 x 10^5 cpm in 50 µl 0.1% BSA-PBS/well). The plates were incubated for 2 hr at room temperature, washed, dried and the wells cut out, transferred into separate tubes and counted in a gamma counter (1227 Gammamaster, LKB, Turku, Finland). Assays were done in triplicates and the results expressed as mean net cpm ± SD, after corrections (1–3%) for non-specific binding in control wells that were coated with BSA and for the binding from the correlate preimmune horse, human and mouse sera

2.5. Immunization of mice with botulinum toxoid or with H_C for T cell studies

(a) *Immunization with toxoid.* Initial experiments were done to determine the optimal toxoid priming dose for the two mouse strains. The mice were immunized s.c. at the base of tail with various doses of toxoid (0.125-5 µg/mouse) in a 50-µl emulsion of equal volumes of the toxoid solution in PBS and CFA containing *Mycobacterium tuberculosis*, strain H37Ra (Difco Laboratories, Detroit, MI). In subsequent studies for mapping of the T cell recognition regions, mice of both strains were immunized in the same manner with 1 µg of toxoid/mouse.

(b) *Immunization with H_C.* In initial experiments, we determined the optimum priming dose for the mouse strains, SJL, BALB/c, C3H/He and C57BL/6. The mice were immunized s.c. at the base of the tail with varying H_C doses (0.25–5 µg/mouse; 3 mice/group) as an emulsion (100 µl) of equal volumes of antigen solution in PBS and CFA containing *Mycobacterium tuberculosis*, strain H37Ra (Difco, Detroit, MI). Epitope mapping was done in SJL and BALB/c mice (10–12 mice/strain), which were immunized in the same manner with H_C (0.25 µg/mouse).

2.6. Immunization of mice with individual peptides or peptide mixtures

Female SJL (H-2^s) and BALB/c (H-2^d) mice, 7–8 weeks old, were immunized with individual peptides (50 µg/mouse) or with an equimolar mixture of peptides (10 µg/peptide/mouse). Three peptide mixtures were used: (1) peptides containing T cell epitopes; (2) peptides containing Ab epitopes; or (3) peptides containing both T cell and Ab epitopes. The peptides were given as an emulsion (100 µl) of equal volumes of CFA containing *Mycobacterium tuberculosis*, strain H37Ra (Difco, MI) and antigen solution in PBS. For the T cell study, mice were injected s.c. at the base of the tail with peptides. For the Ab study in both strains (3 mice/group), peptides were injected i.p. in the foot pads, and booster injections of similar doses in incomplete Freund's adjuvant were given at 3, 7, 11 and 15 weeks. Sera were collected prior to the first injection and at 4, 8, 12 and 16 weeks.

Equal volumes of a given bleed from each group were mixed and kept frozen (at –20°C) until use. Mixtures of preimmune sera from the correlate groups were also prepared in a similar manner.

2.7. Proliferation assay of toxoid-primed, H_C-primed or peptide-primed LNC

(a) *Toxoid-primed LNC.* The inguinal and paraaortic lymph nodes from each group of toxoid-primed mice were harvested and pooled 10 days after the first injection to obtain once-primed LNC or 2 weeks after the last booster injection for the hyperimmune LNC. Single-cell suspensions of LNC, prepared in Hank's balanced salt solution were washed, resuspended, and cocultured, at 5 x 10^5 cells/well, in triplicates in flat bottom microtiter plates (Corning, Corning, NY) with various challenge concentrations of toxoid (0.3-10 μg/ml) in a final volume of 200 μl RPMI 1640 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 2 mM L-glutamine, 10 mM HEPES, 4 x 10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% normal mouse serum. In addition to unstimulated LNC, controls included cells stimulated with Con A (Sigma Chemicals, St. Louis, MO; 1 μg/ml), irrelevant protein (myoglobin; 100 μg/ml) and unrelated synthetic peptide (sequence: ISEAIIHVLHSRHP; 40-80 μg/ml). For mapping the regions of T cell recognition, the cells were challenged with each of the overlapping peptides (20-80 μg/ml), H_C (1.25-5 μg/ml) or toxoid (0.3-10 μg/ml). After incubation at 37°C in a humidified 5% CO_2 atmosphere for 3 days, the cultures were pulsed with 1 μCi/well of [^{3}H]thymidine (ICN Biomedicals, Irvine, CA) for 18 h and then harvested on glass-microfiber filters and assayed for their radioactivity. Results are expressed in net cpm ± SD or in stimulation index (S.I. = average cpm incorporated by stimulated cells/average cpm incorporated by unstimulated cells) ± SD.

(b) H_C-primed lymph node cells. Inguinal and paraaortic LNC were harvested from H_C-primed mice and pooled 7–8 days after the first immunization or the last booster for the hyperimmune LNC. Single-cell LNC suspensions were co-cultured in triplicate as described above at 5 × 10^5 cells/well with various concentrations of H_C (0.15–5.0 μg/ml), BoNT/A peptides (0.5–80 μg/ml). Controls included Con A (1 μg/ml), LPS (500 μg/ml), unrelated proteins (ovalbumin, lysozyme, myoglobin; 100 μg/ml) and unrelated synthetic peptide (sequence: ESSGTGIESSGTGI; 10–40 μg/ml. For the purpose of this study, an S.I. value ≥ 3 was considered to be a positive response.

(c) *Proliferation assay of peptide(s)-primed lymph node cells.* LNC were harvested From each group of peptide(s)-primed mice and pooled 7–8 days after immunization. LNC were co-cultured as above at 5 to 6 × 10^5 cells/well in the presence of the immunizing peptides (0.5–80 μg/ml), H_C (0.15–5 μg/ml), Con A (1–4 μg/ml), LPS (500 μg/ml), control unrelated proteins (ovalbumin, lysozyme, myoglobin; 100 μg/ml) or unrelated synthetic peptide (10–40 μg/ml). Results were expressed in stimulation index at the optimum challenge doses of each antigen.

2.8. Binding assay of anti-peptide(s) Abs to the immunizing peptide and to H_C

Binding of anti-peptide(s) Abs to the immunizing peptide(s) and to H_C was determined by solid-phase RIA as described above for the binding of anti-toxin and anti-H_C antibodies.

3. RESULTS

PART I - THE H_C REGIONS RECOGNIZED BY ANTI-TOXIN ABS FROM THREE HOST SPECIES

3.1. Binding of Horse Anti-BoNT/A Abs to BoNT/A Peptides and to toxoid

The binding results of BoNT/A, H_C and the synthetic peptides with horse anti-BoNT/A Abs are summarized in Fig. 2. The areas that gave positive binding (in decreasing order) were: peptides 31 (residues 1275–1296), 1 (855–873), 30 (1261–1279), the 25/26 (1191–1209/1205– 1223) overlap, 7 (939–957) and the 17/18 (1079–1097/1093–1111) overlap. In addition, peptides 3 (883–901), 5 (911–929), 11 (995–1013), 13/14 (1023–1041/1037–1055) overlap, 22 (1149– 1167) and 26 (1205–1223) gave low but significant and reproducible binding.

3.2. Binding of Human Anti-BoNT/A Abs to Peptides and to toxoid

The human antiserum against BoNT/A was lgG fraction of a pool of antisera from several volunteers who were injected with toxoid. The results of Ab binding to the synthetic peptides and to toxoid at antiserum dilutions of 1:1000 and 1:2000 (vol/vol) are summarized in Fig. 3. High amounts of Abs were bound by peptides 2 (residues 869–887), 6 (925–943), 10 (981–999) at 1:1000 dilution, 11 (995–1013), 15 (1051–1069) and 24 (1177–1195). In addition, lower amounts of Abs were bound by peptides 5 (911–929) at 1:1000 dilution, 7 (939–957), 9 (967–985), 20/21 (1121–1139/1135–1153) overlap and 29/30/31 (1247–1265/1261–1279/1275– 1295) overlap. Peptides 2, 6, 11, 15 and 24 exhibited high binding at both dilutions. On the other hand, peptide 10, which bound high amounts of Abs at 1:1000 dilution, showed little binding when the serum was diluted to 1:2000. It appears, therefore, that the Abs against the 9/10/11 overlap are of lower affinity than the Abs directed against peptides 2, 6, 15 and 24.

3.3. Binding of Outbred Mouse Anti-BoNT/A Abs to Peptides and to toxoid

Mouse anti-toxoid antisera were studied at two dilutions, 1:500 and 1:1000 (vol/vol). The results (summarized in Fig. 4) indicate that mouse anti-toxoid Abs recognize essentially six antigenic regions that fall (in decreasing order of immunodominance) within peptides 2/3 (residues 869–887/883–901) overlap, 15 (1051–1069), 24 (1177–1195), 31 (1275–1296), 11 (995–1013) and 7 (residues 939–957), and. Additionally, two very minor antigenic sites might occur within peptides 18 (1093–1111) and 20/21 (1121–1139/1135–1153) overlap.

Table 1 summarizes the binding profiles of anti-toxoid Abs from the above three species.

PART II - THE H_C REGIONS RECOGNIZED BY MOUSE ANTI-TOXOID T CELLS AND ABS

3.4. Binding of anti-toxoid antibodies to the overlapping peptides and to toxoid

The hyperimmune anti-toxoid antisera possessed similar Ab titers in their reactivity with toxoid (Fig. 5). For mapping of the Ab binding profile to the peptides, the antisera were used at dilution 1:500 (v/v). The binding profiles of anti-toxoid Abs from BALB/c and SJL were similar (Fig. 6 and 7). For BALB/c, anti-toxoid Abs were bound mainly by peptides 24 (residues 1177–1195), which

was strongly immunodominant, the 2/3 overlap (869–887/883–901), 21 (1135–1153) and 31 (1275–1296). In addition, lower, but significant amounts of Abs were bound by peptides 11 (residues 995–1013) and 15 (residues 1051–1069). The other peptides exhibited marginal or no Ab binding activity. The anti-toxoid Abs of SJL (Fig. 7) recognized five antigenic regions within peptides 2/3 overlap (residues 869–887/883–901), 11 (995–1013), 15 (1051–1069), 24 (1177–1195) and 31 (1275–1296). The only noticeable difference in the binding profiles of anti-toxoid Abs from BALB/c and SJL mice was connected with their ability to recognize peptides 11, 15 and 21. Unlike BALB/c Abs, which exhibited low binding to peptides 11 and 15, the anti-toxoid Abs of SJL displayed high binding activity to both peptides. On the other hand, peptide 21 bound higher amounts of anti-toxoid Abs of BALB/c than did those of SJL antisera.

3.5. Proliferative response of toxoid-primed LNC to challenge with toxoid

Experiments were first carried out to determine the optimum *in vivo* priming and *in vitro* challenge doses of toxoid. We varied the *in vivo* priming doses of toxoid from 0.125 to 5 μg/mouse and the *in vitro* challenge concentration of toxoid from 0.3 to 10 μg/ml. Figure 8 summarizes the results of these studies in BALB/c mice. The highest response was given by T cells from mice that received a priming dose of 1 μg/mouse. T cells of mice injected with the lowest dose of 0.125 μg/mouse did not respond to toxoid but responded strongly to *in vitro* stimulation with Con A indicating their viability. The dose-dependent profiles of T cell responses from toxoid-primed SJL mice were similar to those obtained for BALB/c. The highest T cell response in SJL was also achieved at a priming dose of 1 μg/mouse. Based on these results, a toxoid priming dose of 1.0 μg/mouse was used in all subsequent experiments for both mouse strains.

3.6. Mapping of the T cell recognition profiles after one injection with toxoid

Following priming with a single injection of toxoid, the proliferative responses of T cells were determined *in vitro* to various H_C challenge doses (1.25-5 μg/ml) and each of the synthetic peptides (20-80 μg/ml). The toxoid-primed LNC of BALB/c responded strongly to challenge with H_C (S.I. 24.79 ± 0.29) and also to peptides 4 (S.I. 4.42 ± 0.13), 7 (S.I. 4.35 ± 0.01) and 12 (S.I. 2.29 ± 0.11) (Fig. 9). The cells did not respond to the other peptides at any of the concentrations tested. Also, unrelated protein (myoglobin) and peptide were totally non-stimulatory. The viability of cells was confirmed by their strong response to Con A (S.I. 20.64 ± 0.65). In contrast to BALB/c, the toxoid-primed LNC from SJL mice exhibited a significant difference in their ability to proliferate in response to challenge with peptides. The recognition profile of T cells from toxoid-primed SJL mice (Fig. 10) revealed that, within region 897–971 (overlapping peptides 4 to 8), two peptides, 4 and 7, were notably immunodominant (S.I. values of 12.47 ± 0.10 and 5.30 ± 0.19, respectively) and were also recognized by T cell of BALB/c (Fig. 9). Moreover, toxoid-primed T cells of SJL proliferated very well (S.I. 6.05 ± 0.27) in response to *in vitro* challenge with peptide 15 (residues 1051–1069) and showed low, but still significant responses to peptides 12 to 25 (1009–1209), 28–29 (1233–1265) and 31 (1275-1296). The cells responded very strongly to H_C (S.I. 29.99 ± 0.15) but

did not respond to the remaining peptides (1–3, 9–11, 26–27 and 30), unrelated peptide or myoglobin at any concentrations tested. Their viability was evidenced by their vigorous response to Con A (S.I. 28.50 $\pm$ 0.30). Table 2 compares the recognition profiles of anti-toxoid Abs and T cells from the two strains.

3.7. Mapping of the T cell recognition profiles after multiple injections with toxoid

We also examined the proliferative responses of LNC obtained from BALB/c and SJL mice that were used to prepare hyperimmune anti-toxoid antisera for the Ab binding studies. LNC were harvested at the time of the final bleed on week 10, i.e. 2 weeks after the last of three injections of toxoid. The proliferative responses of LNC from once-primed (group 1) and from hyperimmune (group 2) BALB/c and SJL mice are compared for each strain in Table 3. For BALB/c, these two recognition profiles were slightly different. Hyperimmune T cells responded to *in vitro* challenge with peptides 4 (residues 897-915), 6 to 8 (residues 925–971), 22 (residues 1149–1167) and very well to peptide 30 (residues 1261–1279). For SJL, the recognition profiles of once-primed and of hyperimmune LNC also showed some differences (Table 3), mostly indicative of slight shifts in the epitope recognition pattern. Even though, a number of overlapping peptides possessed the capacity to induce the proliferative response of toxoid-primed LNC from both groups of SJL mice, three peptides were consistently the most potent T cells stimulators. Peptide 4 (residues 897–915) was prominently immunodominant in both groups 1 and 2. The *in vitro* proliferative response of SJL hyperimmune LNC to peptide 4, however, increased remarkably and reached nearly double that given by the once-primed cells (see Table 3). The hyperimmune LNC of SJL responded better to peptides 6 (residues 925–943) and 30 (residues 1261–1279) than did the once-primed cells. The immune T cells from SJL mice of both groups also responded strongly to peptide 15 (residues 1051–1069) and moderately to peptides 7 (residues 939–957) and 20 (residues 1121–1139). The remaining peptides (Table 3) evoked low or moderate proliferative responses.

PART III – THE H_C REGIONS RECOGNIZED BY MOUSE ANTI-H_C ABS AND T CELLS

3.8. Binding to peptides of anti-H_C. Abs from SJL and BALB/c mice

The antisera obtained 12 weeks after the initial injection were used in the mapping studies with the synthetic peptides. Binding studies, to H_C and to peptides, with different dilutions of anti-H_C antisera showed that, for both SJL and BALB/c antisera, maximum binding was obtained at dilutions between 1:500–1:1000 (vol/vol). A dilution of 1:500 (vol/vol) was used in all subsequent binding studies. The results of mapping of anti-H_C antisera from SJL and BALB/c mice are summarized in Fig. 11. Both antisera showed high levels of Abs that were bound by H_C. There were similarities in the Ab binding profiles of SJL and BALB/c anti-H_C antisera but the two profiles were not identical (Fig. 11 and Table 4). With SJL anti-H_C antisera, Ab responses were immunodominant against peptide 31 (residues 1275–1296) followed by peptides 4 (residues 897–915), 24 (residues 1177–1195), 7 (residues 939–957), 11 (residues 995–1013) and 15 (1051–1069). Peptides 1/2/3

(residues 855–901) and 18/19 (residues 1093–1125) exhibited intermediate Ab binding, while binding to peptides 10 (residues 981–999) or 6 (residues 925–943) was low. Very little or no significant binding to the remaining peptides was observed. BALB/c anti-H_C Abs showed very high binding to peptides 31 (residues 1275–1296), 24 (residues 1177–1195), 2 (residues 869–887) and 17/18 (residues 1079–1111), followed by high binding to peptides 3 (residues 883–901) and 10 (residues 981–999), and an intermediate response against peptide 21 (residues 1135–1153). Low amounts of Ab were bound by peptides 9 (residues 967–985), 15 (residues 1051–1069), 6/7 (residues 925–957), 1 (residues 855–873) and 11 (residues 995–1013). The remaining peptides showed negligible or no binding. It should be noted that neither SJL nor BALB/c anti-H_C antisera were bound by protein and peptide controls that were unrelated to BoNT/A.

3.9. Mouse strains and determination of optimum H_C priming and challenge doses.

To identify mouse strains that are high responders to H_C, we examined the responses to the latter of four different independent mouse haplotypes, SJL (H-2^s), BALB/c (H-2^d), C3H/He (H-2^k) and C57BL/6 (H-2^b). We determined the optimum dosage conditions to obtain the maximum responses in each mouse strain. In each of these mouse strains, we varied the *in vivo* H_C priming doses from 0.25 to 5 μg/mouse and the *in vitro* challenge doses from 0.08 to 10 μg/ml. The T cells of H_C-primed SJL mice responded very strongly to H_C at all 4 priming doses. T cells of H_C-primed BALB/c mice also responded strongly to H_C, giving the highest response at the lowest priming dose of 0.25 μg/mouse. In contrast, C3H/He and C57BL/6 mice were low responders to H_C, in comparison to SJL and BALB/c mice. The mapping studies were, therefore, done in SJL and BALB/c using H_C-priming doses of 0.25 or 0.5 μg/mouse for SJL and 0.25 μg/mouse for BALB/c. At these H_C-priming doses, LNC gave very low responses to H_C challenge doses of 10 μg/ml and 0.08 μg/ml. In all subsequent studies, H_C was employed at challenge doses of 0.15, 0.3, 0.6, 1.2, 2.5 and 5 μg/ml of culture.

3.10. Proliferative response of H_C-primed T cells to challenge with peptides

In T-cell proliferative studies, H_C-primed LNC of SJL responded very strongly to challenge with each of the overlapping peptides 4, 5, 6, 7, 8 and 9 spanning residues 897–985. The cells also mounted a strong proliferative response to challenge with peptide 15 (residues 1051–1069). On the other hand, Peptides 11 (residues 995–1013), 14 (residues 1037–1055) and 20 (residues 1121–1139) evoked weak but significant responses, while remaining H_C peptides evoked no significant responses (Fig. 12). The optimum *in vitro* stimulating dose varied with the peptide. The cells responded very strongly to H_C (Fig. 12) but not to proteins and peptides that are unrelated to BoNT/A (ovalbumin, lysozyme, myoglobin and unrelated peptide).

The H_C-primed T cells from BALB/c proliferated, in decreasing order of response levels, to peptides 21 (residues 1135–1153), 7 (residues 939–957) and 12 (residues 1009–1027). The remaining peptides did not stimulate any significant responses in BALB/c. The cells proliferated strongly to H_C challenge and gave no response to unrelated proteins and peptide (Fig. 12).

Hyperimmune LNC were obtained from SJL and BALB/c mice at the time of the final bleeding for the hyperimmune anti-H_C antisera (i.e., one week after 5 booster injections in SJL and 4 injections in BALB/c). The recognition profile of H_C-primed SJL hyperimmune LNC was very similar to that described above for T cells obtained after one H_C injection. Hyperimmune T cells of H_C-primed SJL mice responded to peptides 4–9, with the response to peptide 7 (residues 939–957) being the strongest. There was, however, a slight shift in the region recognition around the overlapping peptides 15 and 16. Cells from hyperimmune SJL mice mounted a significant response to challenge with peptide 16 (residues 1065–1083), whereas those obtained at one week (after one H_C injection) responded to peptide 15 (residues 1051–1069). The remaining peptides did not evoke any significant responses. Hyperimmune BALB/c T cells gave significant responses, in decreasing order, to peptides 21 (residues 1135–1153), 15 (residues 1051–1069), 5 (residues 911–929), the 7/8 overlaps (residues 939–971) and 2 (residues 869–887). The remaining peptides stimulated no significant responses. Thus, in BALB/c, there were some differences in the T-cell recognition profile in hyperimmune mice but the responses to peptides 21 (residues 1135–1153) and 7 (residues 939–957) persisted on long-term immunization.

PART IV. T CELLS AND ABS AGAINST H_C PEPTIDES OF AND THEIR CROSS-REACTION WITH H_C

3.11. T cell proliferative response after immunization with the individual peptides

The results of T-cell proliferative assays for peptide immunizations are summarized in Table 4. T cells against the following peptides proliferated *in vitro* to the immunizing peptide (in decreasing order of magnitude of response): SJL, peptides 4, 7, 8, 5, 6, 10, 15 and 31; BALB/c, peptides 7, 21, 12, 2, 24 and 31. In SJL, priming with peptides 4 through 8 and 10 induced cells that were responsive *in vitro* to H_C (S.I. $\geq$ 3.0), but the magnitude of these responses varied. In BALB/c mice, only the responses to peptides 7 and 12 could recognize H_C.

3.12. Binding to peptides and to H_C of Abs from mice immunized with individual peptides

Based on the initial titration study using dilutions of 1:200 to 1:8000, a dilution of 1:500 (vol/vol) was used in all binding studies with bleeds at 4, 8, 12 and 16 weeks. Maximum Ab binding to the immunizing peptide and to H_C of each of the anti-peptide antisera at the optimal bleeds is also presented in Table 5. In SJL, the following peptides stimulated Ab responses (in decreasing order): peptides 10, 4, 6, 7, 5, 8, 11, 24, 31 and 15. Antibodies against peptide 4 showed a very high level of cross-reactivity with H_C, followed by peptide 10. In BALB/c, all the peptides tested generated Abs to the respective peptides and these Abs cross-reacted with H_C. However, the levels of reaction with the homologous antigens (peptides) and with H_C varied. Among these, Abs against peptide 31 showed the highest binding to H_C. Thus, peptide 4 in SJL and peptide 31 in BALB/c generated Abs that gave the highest cross-reactions with H_C. The results of time-course study of Ab binding to H_C of the antisera generated against each of these two peptides are given in Fig. 13. Peptide 4 (in SJL) generated maximum reaction with H_C at 8 weeks, and peptide 31 (in BALB/c) at 12 weeks. Other

peptides showed their highest cross-reactive titers to H_C at 8–16 weeks (after 3–5 injections)(data not shown).

3.13. T cell responses against the peptide mixtures

We used three different mixtures of peptides containing: (a) epitopes recognized by anti-H_C T cells; (b) epitopes recognized by anti-H_C Abs; or (c) T cell + Ab epitopes. Each peptide mixture was used to prime two mouse strains. The LNC from the SJL mice that had been primed with each of the three peptide mixtures responded to challenge *in vitro* with H_C in the following order: mixture (c) primed T cells (S.I.=115), mixture (a) primed T cells (S.I.=33), and mixture (b) primed T cells (S.I.=17). Mixture-primed LNC of BALB/c responded *in vitro* to Hc in the following order: mixture (c) group, (S.I.=22), and mixture (a) primed T cells, (S.I.=19), while mixture (b) primed T cells gave no response to H_C. Thus in both strains, mixture (c) (composed of peptides that contained T cell and Ab epitopes when H_C was the immunogen) gave the highest proliferative response to H_C. The results of proliferation to individual peptides of T cells that had been primed with mixture (c) are given in Table 6. Challenge with the following peptides evoked positive responses (in decreasing order): SJL, peptides 4, 7, 3, 10, 15, 9, 31, 6, 8, 5 and 24; BALB/c, peptides 12, 7 and 21.

3.14. Binding to peptides and to H_C of Abs from mice immunized with peptide mixture.

The time-course study results of binding to H_C of Abs from 2 mouse strains against a mixture of T and/or Ab epitope-containing peptides are presented in Fig. 13. The highest cross-reactions with H_C for Abs against each peptide mixture varied and were obtained at 4 to 16 weeks (after 2–5 injections). Among the three mixture groups tested, the group composed of peptides that contained T cell and Ab epitopes generated the highest cross-reaction with H_C for both strains at 4 weeks (after 2 injections), and, in the case of BALB/c, sustained a high titer at 8–16 weeks. The Ab binding to each of the constituent peptides in the antisera against this peptide group is summarized in Table 6. Antibodies were bound by the following peptides (in the decreasing order): SJL, peptides 4, 10, 31, 3, 7, 9, 8, 2, 24, 6, 11 and 5; BALB/c, peptides 31, 10, 24, 2, 3, 7 and 18.

4. DISCUSSION

Among the various strategies for developing new vaccines, the use of synthetic peptides has great appeal in both conceptual simplicity and feasibility of large-scale production. Vaccines based on peptide design are safe, cheap, stable, easy to store and handle, and ideally suited to targeting for specific purposes. The strategy is based on the preparation of synthetic peptides that mimic protective epitopes of the pathogen. The development of a synthetic vaccine against BoNTs will be a multistep process. The first prerequisite is the knowledge of the structure of the toxins. Another requirement is the detailed knowledge of the T and B cell recognition of the antigen.

When this work started very little was known about the submolecular T and B cell recognition of BoNTs. The lack of structural information on BoNTs, permitted analysis of their immune

recognition only at the level of the subunits (50 and 100 KD) or of relatively large fragments (50 KD) (Kozaki *et al.*, 1986, 1989; Tsuzuki *et al.*, 1988; Chan *et al.*, 1993). In contrast, the immune recognition of TeTX, another clostridial neurotoxin whose primary structure was determined earlier (Eisel *et al.*, 1986; Fairweather and Lyness, 1986), has been investigated in more detail (Demotz *et al.*, 1989; Ho *et al.*, 1990; Reece *et al.*, 1993; Fischer and Howden, 1994). Recently, However, the complete primary structures of BoNT types A (Binz *et al.*, 1990a; Thompson *et al.*, 1990), B (Jung *et al.*, 1992; Whelan *et al.*, 1992a), C1 (Hauser *et al.*, 1990), D (Binz *et al.*, 1990b), E (Poulet *et al.*, 1992; Whelan *et al.*, 1992b), F (East *et al.*, 1992) and G (Campbell *et al.*, 1993) have been determined. In addition the disulfide pairing in BoNT/A has been established (Krieglstein *et al.*, 1990, 1994). BoNT/A has been crystallized and a preliminary X-ray analysis of its structure has been reported (Stevens *et al.*, 1991).

As already mentioned in the Introduction, immunization with H_C of type A afforded excellent protection against BoNT/A poisoning (Middlebrook, 1995; Clayton *et al.*, 1995). Therefore, the immunological mapping H_C would be extremely valuable for the eventual design of a synthetic anti-BoNT vaccine. Our plan was done in the following stages: (1) To map the *continuous* recognition regions with Abs and T cells against BoNT/A and against H_C. (2) The peptides that contain Ab and/or T cell epitopes would then be used as immunogens to identify anti-peptide Ab and/or T cell responses that cross-react with H_C. Although the cross-reaction of an anti-peptide response with the parent molecule does not imply protection, it is an essential requirement for it; and (3) To determine, in the final stage, the protective ability of the peptides identified in (2).

4.1. Antibody recognition regions in the C-terminal domain after immunization of different host species with BoNT

Several regions of H_C were recognized by horse, human and mouse anti-BoNT/A Abs. Comparison of the peptide binding profiles for horse, human and mouse Abs revealed considerable similarities (see Fig. 2, 3 and 4, and the summary in Table 1). Both human and mouse antisera recognized peptides 2, 15 and 24. With horse antiserum, both the first and second epitopes were shifted to the left and resided within peptides 1 and 13/14, respectively, while the third was shifted to the right and resided within the 25/26 overlap. A region recognized by the human antisera within the overlap of peptides 5/6/7 was more weakly recognized and shifted in favor of peptide 7 in the mouse antisera. In horse antiserum, both peptides 5 and 7 (but not 6) were recognized. The lack of recognition of peptide 6 suggests that this region harbors two epitopes that can be distinctly resolved by the horse, but not by the human and mouse, antisera with the present panel of peptides. The human antisera recognized a region within the overlap 10/11. This region was also recognized by mouse, and more weakly by horse, antisera and was shifted to the right towards peptide 11. Peptide 18 was well recognized by horse, weakly by mouse and not at all by human antisera. A very weak region was recognized by all three antisera around the overlap 20/21 (human and mouse) or 20/21/22 (horse). A broad region recognized within peptides 29/30/31 by human antisera and within

30/31 by horse antisera was more sharply localized within peptide 31 by the mouse antisera. In addition, to these shifts there were differences in immunodominance of the peptides recognized by antisera of the three species. It has been well established that the antigenic sites on a given protein may show boundary frame shifts and may also vary in immunodominance, depending on the host species in which the Abs are raised. These variations may even occur among individual animals of the same host species (Atassi, 1975, 1980, 1984). These results are consistent with genetic control operating at the antigenic site level. It is well established that the immune responses to proteins are controlled by H-2 linked genes (Okuda *et al.*, 1978; Rosenwasser *et al.*, 1979; Krco and David, 1981). In the immune responses (both Ab and T cell) to a multi-determinant complex protein antigen, the responses to each determinant are under separate genetic control (Okuda *et al.*, 1979; Twining *et al.*, 1981; David and Atassi, 1982).

It is necessary for a successful design of a synthetic vaccine to take into account both Ab and T-cell recognition epitopes. Thus, having determined, in horse, human and outbred mouse, the regions on H_C that are recognized by anti-BoNT/A Abs, we then determined the Ab and T-cell recognition regions in BALB/c and SJL after immunization with toxoid or with H_C.

4.2. Antibody and T cell recognition regions after immunization with toxoid

Following immunization of SJL ($H-2^s$) mice (very high responders to toxoid) with pentavalent toxoid, their LNC proliferated very strongly to *in vitro* challenge with recombinant H_C and also gave strong-to-moderate responses to a number of H_C peptides (see Fig. 10). Three peptides, 4 (897–915), 7 (939–957) and 15 (1051–1069) were notably immunodominant and consistently evoked strong proliferative T cell responses. Among these three peptides, the epitope within region 897-915 (peptide 4) was clearly prominent. Immunodominance of this region was even more apparent after repeated immunization of SJL mice with toxoid (see Table 3). The proliferative response of SJL hyperimmune LNC (group 2) to challenge with peptide 4 increased considerably and reached nearly double that obtained with LNC from mice (group 1) that received single injection (see Table 3). Toxoid-primed LNC of BALB/c ($H-2^d$) mice (high-to-moderate responders), on the other hand, responded well to stimulation with H_C but exhibited a more restricted recognition pattern. These cells were able to proliferate in response to challenge with peptide 4 (897–915) or 7 (939–957), and barely responded to stimulation with peptide 12 (1009–1027) (Fig. 9 and Table 3). After two booster injections with toxoid, immune T cell of BALB/c increased their recognition capacity and responded extremely well to challenge with peptide 30 (1261–1296) and moderately to peptide 22 (1149–1167). Clearly, the Ir genes of the mice influenced the recognition profiles of their T and B cells. It was demonstrated previously, that in the case of multi-epitope protein antigens, the Ab and T cell responses to different epitopes on a given protein are controlled by distinct H–2 linked Ir genes (45-48). Our data reported here are in full agreement with these observations.

Comparison of the submolecular T cell recognition profiles of toxoid-primed LNC obtained from the two inbred mouse strains revealed two distinct types of T cell recognition sites on H_C. Some epitopes were unique for a given strain of toxoid-primed mice, while other epitopes were recognized by both strains, irrespective of their MHC haplotype. For example, toxoid-primed LNC of BALB/c mice failed to respond to challenge with peptide 15, while in SJL mice this peptide is one of the three most potent T cell stimulators. On the other hand, epitopes located within regions 897–915 (peptide 4) and 939–957 (peptide 7) were recognized by toxoid-primed LNC of both strains. Peptides that appear to have general recognition across MHC haplotypes would be advantageous for a universal synthetic vaccines as they would be functional in many individuals. It is relevant to mention that the results presented here were obtained with toxoid-primed LNC, i.e. unselected Th cells. Such data may be more useful for design of a synthetic vaccine than those established from study of the best-growing T cell clones (Atassi, 1984; Atassi and Bixler, 1988)

In contrast to the T cell responses, the differences between the B cell recognition profiles of the two mouse haplotypes were less pronounced. Several regions recognized by Abs were similar, although the level of Abs to a given region varied with the strain (see Fig. 6 and 7). Both BALB/c and SJL anti-toxoid Abs exhibited high binding particularly to region 1177-1195 (peptide 24) followed, in decreasing levels of reactivity, by regions 869–887/883-901 (overlap 2/3) and 1275-1269 (peptide 31). In addition to these regions that are recognized by Abs of both strains, a significant amount of BALB/c anti-toxoid Abs was bound by peptide 21 (1135–1153). Unlike BALB/c antisera, which contain very low levels of Abs that bind to regions 995-1013 (peptide 11) and 1051-1069 (peptide 15), those of SJL displayed high Ab binding to both regions.

4.3. Antibody and T cell recognition regions after immunization with H_C

Our studies showed that, at the T-cell level, SJL (H-2^s) and BALB/c (H-2^d) mouse strains are very high and high responders, respectively, to H_C. Whereas, C3H/He (H-2^k) and C57BL/6 (H-2^b) strains are low responders. Therefore, we decided to map, in the SJL and BALB/c mouse strains, the *continuous* regions recognized by T-cell and Ab responses against H_C. In the two independent mouse haplotypes, the immunodominance of various BoNT/A regions varied with the haplotype (Fig. 13), which is again indicative of genetic control operating at the antigenic site level.

H_C-primed T cells of BALB/c recognized three regions residing within residues 937–957 (peptide 7), 1009–1027 (peptide 12) and 1135–1153 (peptide 21). The response to region 1135–1153 was immunodominant at one week (Fig. 13) and persisted in the long-term immunization (see Results). The regions recognized strongly by T cells from H_C-primed SJL mice clustered in a large area within residues 897–985 comprising the overlapping peptides 4, 5, 6, 7, 8 and 9 in the first N-terminal third of H_C. There was only one additional region within residues 1051–1069 (peptide 15) which stimulated a moderate response in these T cells. The crowding of the regions recognized by SJL T cells to the first third of the H_C is unusual and its significance (in terms of protection) in this strain will be the subject of further investigation. Dose-response curves

showed that peptide regions 897–915 (peptide 4) and 939–957 (peptide 7) can be recognized by T cells even at the lower concentrations (0.6–10 μg/ml) of these peptides, while higher challenge concentrations (≥ 80 μg/ml) were needed to achieve optimal responses by peptides 5, 6, 8 and 9, suggesting that this cluster might consist of at least two immunodominant regions around residues 897–915 and 939–957. Among these two immunodominant peptides, immunodominance of region 939–957 persisted in the long-term immunization as revealed by the examination of hyperimmune LNC (see Results). T cells of both SJL and BALB/c mice recognized region 939–957 (peptide 7) (Fig. 13), indicating that this region of H_C can bind different MHC class II alleles. Promiscuous T cell epitopes that can be recognized by different MHC class II molecules might be beneficial for a universal vaccine, since human recipients of the vaccine possess different MHC class II haplotypes.

It has been reported (Corradin *et al.*, 1990; Demotz *et al.*, 1989) that in TeTX, region 947–967 is recognized by human peripheral blood lymphocytes. This region of TeTX is homologous to BoNT/A region 938–958 which is represented by peptide 7 (residues 939–957) and is found in the present work to be recognized by SJL and BALB/c T cells. Region 916–932 of TeTX, equivalent to BoNT/A region 907–923 that is encompassed by the overlap of peptides 4 (residues 897–915) and 5 (residues 911–929) and recognized by SJL T cells, has also been found to be recognized by human T cells (Ho *et al.*, 1990). These similarities in T-cell recognition regions indicate that BoNT and TeTX share some of immunological features at the T cell level, along with a number of structural and functional similarities. It has been well established that, in closely related proteins, the sites of immune recognition often occur at structurally equivalent locations (Kazim and Atassi, 1977; Atassi and Kazim, 1978; Kazim and Atassi, 1982; Atassi, 1984).

While H_C-primed LNC from SJL and BALB/c recognized a common as well as different epitope regions on H_C, recognition regions by Abs essentially overlapped, although the level of Abs to each region differed between the two strains (summary in Table 4). There were seven common or similar regions (4 common; 3 similar) of recognition in the two strains. They are: residues 855–915 (peptides 1–4) for SJL or residues 855–901 (peptides 1–3) for BALB/c; residues 925–957 (peptides 6–7) for both strains; residues 981–1013 (peptides 10–11) for SJL or residues 967–1013 (peptides 9–11) for BALB/c; residues 1051–1069 (peptide 15) for both strains; residues 1093-1125 (peptides 18-19) for SJL or residues 1079-1111 (peptides 17-18) for BALB/c; residues 1177–1195 (peptide 24) for both strains; and residues 1275–1296 (peptide 31) for both strains. In addition to these areas, Abs from BALB/c recognized region 1135–1153 (peptide 21). Similar observations were recently made in SJL and C57BL/6 mice primed with AChR (52), in which major Ab recognition regions for both strains were clustered into 3 similar regions in α1–210 of AChR α chain, whereas, T cells from each strain recognized different peptide regions.

Table 4 summarizes the results of peptides recognized, in SJL and BALB/c strains, by anti-H_C Abs and/or T lymphocytes. Comparison of the profiles of the Ab and T-cell responses in the same mouse strains revealed that, in a given mouse strain, certain regions are recognized by both Abs and T cells. In SJL, both Abs and T cells recognized regions 897–915 (included in peptide 4), 939–957

(peptide 7) and 1051–1069 (peptide 15), whereas in BALB/c regions 939–957 (peptide 7) and 1135–1153 (peptide 21) were recognized by both Abs and T cells. There were, however, regions that were predominantly recognized either by Abs or by T cells. The results of Ab and T cell recognition of H_C are consistent with observations (mentioned above) on immunization with or other protein antigens.

4.4. T cell and Ab anti-peptide responses and their cross-reaction with H_C

In order for anti-peptide responses to be protective it is obligatory that such responses recognize and cross-react with H_C. Of course, not every anti-peptide response is expected to cross-react with H_C and, furthermore, those that do may not all be protective. The purpose of this study was to identify the immunodominant H_C regions, which stimulate immune T cell and Ab responses that are able to cross-react with intact H_C. Peptides containing Ab and/or T cell epitopes (when H_C is the immunogen) were immunized individually or in equimolar mixtures.

The results of immunization with individual peptide are summarized in Table 5. In BALB/c, all the peptides that contained Ab and/or T-cell epitopes [when H_C is the immunogen (Oshima *et al.*, 1996)] produced Ab responses to both immunizing peptides and to H_C. Strong H_C-cross-reactive Abs were generated by peptides 2, 3, 10 and 31 which contain epitopes recognized by anti-H_C Abs (Oshima *et al.*, 1996). In SJL, anti-peptide Abs were elicited by most of the peptides that contain Ab and T epitopes. However, a very strong H_C-cross-reactive Ab was obtained by peptide 4 followed by peptide 10. The greater immunogenicity of peptide 4 in SJL might be rationalized by the fact that it contains both T and B cell epitopes. T cell responses that were cross-reactive with H_C were elicited by peptides 4–8 and 10 in SJL, and peptides 7, 12 and 17 in BALB/c. Except for peptide 17 in BALB/c, each of these peptide-primed T cells showed moderate to very strong proliferative response to the immunizing peptide. However, T cells against peptides 2, 21, 24 and 31 in BALB/c showed negligible response to challenge with H_C (Table 5). This is consistent with previous observations (Bixler and Atassi, 1985; Bixler *et al.*, 1985; Bhardwaj *et al.*, 1994; Hoyne *et al.*, 1993) with anti-peptide T cells that failed to recognize the parent protein.

Among the three groups of peptide mixtures, the one of peptides containing both Ab and T cell epitopes was most effective in both strains in eliciting T cells and Abs that were cross-reactive with H_C (see Results and Fig. 13). There were qualitative and quantitative differences in the peptide recognition profile after immunization with this peptide mixture as compared to those obtained with individual peptide immunization (Tables 5 and 6). Immunization with this mixture elicited Abs to some peptides that were otherwise unable to evoke Ab responses when used individually as immunogens (peptides 2, 3 and 9 in SJL). Also, it suppressed Ab responses to certain peptides that could otherwise elicit Abs when injected individually (peptides 12, 17 and 21 in BALB/c). Clearly, help and inter-site influences of the cellular responses against the constituent peptides modulate Ab production to these regions in the peptide mixture. It has been shown that immune responses to various epitopes on an antigen are subject to inter-site T-T and T-B cell interactions (Atassi *et al.*,

1981; Bixler and Atassi, 1985; Bixler *et al.*, 1985; Rosenberg *et al.*, 1996). These interactions and co-immunization effects contribute to the complex responses of T cells and Abs obtained after peptide mixture immunization.

Injection with the peptide mixture containing Ab and T cell epitope peptides (when Hc is the immunogen, Oshima *et al.*, 1996) gave a quicker rise (after 2 injections, at 4 weeks) in Ab titer that cross-reacted with H_C compared to the other mixtures or to individual peptides (Fig. 13). Also, this mixture sustained the high titer of H_C-cross-reacting Abs in the case of BALB/c (Fig. 13). Thus, immunization with a mixture of peptides containing all T and B cell epitopes was particularly effective in BALB/c mice. The results suggest that inclusion of the T cell-epitope containing peptides into the vaccine formula should provide help for and enhance the production of Abs that recognize H_C. It has recently been shown that a mixture of three peptides from α-bungarotoxin was a more protective immunogen against toxin poisoning than each of its constituent peptide individually (Dolimbek and Atassi, 1996).

Sequence alignment of the 17 peptide regions used in this study between BoNT/A, BoNT types B through G and TeTX revealed that thirteen peptides (Fig. 14) have 5 or more continuous residues that are identical or similar to type A in one or more of these clostridial toxins. Of these, peptides 2, 3, 7, 10, 12, 15, 18, 24 and 31 were shown to generate Abs that are cross-reactive with H_C in either strain (Table 5). Addition to the mixture that comprised peptides containing Ab epitopes of T epitope peptides 7 and 12 which contain T cell epitopes and which have identical or similar regions in most of the toxins listed augmented production of Abs that are cross-reactive with H_C in BALB/c (Fig. 13). These results suggest that one or more of these synthetic peptides provides help that might contribute to cross-protection against those toxins. Peptide 7, a T and/or Ab epitope containing peptide for both strains, is immunogenic at both the T and B cell levels in each strain when used as immunogen either individually or in a mixture (Tables 5 and 6). It also generated T and Ab responses that were cross-reactive with H_C (Table 5). It should be noted that region 947–967 of TeTX, similar region to peptide 7 (residues 939–957) is also a universal human T epitope region for TeTX (Demotz *et al.*, 1989). The fact that peptide 7 is effective in both strains suggests that it needs to be included in the design of synthetic vaccines that will be active across MHC haplotypes.

5. CONCLUSIONS

We have determined in work supported by this contract, in horse, human and outbred mouse, the regions on H_C that are recognized by anti-BoNT/A Abs. We also determined the Ab and T-cell recognition regions in BALB/c and SJL after immunization with toxoid or with H_C. Upon immunization with toxoid, certain H_C regions on were recognized by both, Abs and T cells. Such T/B sites were identified within regions 939–957 (peptide 7) in both strains. Additionally, SJL recognized three T/B epitopes located within regions 1051–1069 (peptide 15), 1177–1195 (peptide 24) and 1275–1296 (peptide 31). There were also regions on H_C that were recognized only by T

cell, since no detectable Abs were directed toward these sites. One such exclusive T cell epitope, recognized in both mouse strains but particularly prominent in SJL mice, resided within region 897–915 (peptide 4). Finally, there were regions on H_C that were recognized only by Abs and for which no T cell responses were detected. Two exclusively B cell determinants, common for both strains, were found within regions 869–887/883–901 (overlap 2/3) and 995–1013 (peptide 11).

Following immunization with H_C, anti-H_C Abs from SJL and BALB/c, recognized seven common or similar regions: 855–901/855–915, 939–957, 967–999 /995–1013, 1051–1069, 1079–1111/1093–1111, 1177–1195 and 1275–1296. In addition, BALB/c Abs recognized a region within residues 1135– 1153. The recognition by H_C-primed T cells of SJL clustered within residues 897–985 which probably consists of two major regions within residues 897–915 and 939–957, and a minor region within residues 1051–1069. H_C-primed T cells of BALB/c recognized one immunodominant region within residues 1135–1153 and two other regions within residues 939–957 and 1009–1027. The mapping of the regions recognized by T cells and/or by Abs after immunization with toxoid or with H_C was a crucial step for the design of a synthetic vaccine. Protective immune responses are expected to be directed against T and B cell epitopes within these regions. Accordingly, we studied the T and B cell immune responses that are generated by immunization with these peptides and determined their cross-reaction with H_C.

Several BoNT/A peptides gave immune (Ab and/or T cell) responses that cross-react with H_C. These are peptides 2, 3, 7, 10, 12, 17, 18, 21, 24 and 31; and for SJL they are peptides 4, 5, 6, 7, 8, 10, 15, 24 and 31. The next phase of the work was to incorporate appropriate combinations of these into the formulation of a multi-peptides synthetic vaccine. But this phase was not funded.

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7. PUBLICATIONS

Mapping of the Antibody-Binding Regions on Botulinum Neurotoxin H-Chain Domain 855–1296 with Anti-Toxin Antibodies from Three Host Species.

M. Zouhair Atassi, Behzod Z. Dolimbek, Makoto Hayakari, John L. Middlebrook, Bruce Whitney, and Minako Oshima

J. Prot. Chem. (1996) **15,** 691–700.

Localization of the Regions on the C-Terminal Domain of the Heavy Chain of Botulinum Toxin A Recognized by T Lymphocytes and by Antibodies After Immunization of Mice with Pentavalent Toxoid.

Jana S. Rosenberg, John L. Middlebrook and M. Zouhair Atassi

Immunological Investigations (1997), in press.

Immune Recognition of Botulinum Neurotoxin Type A: Regions Recognized by T Cells and Antibodies against the Protective H_C Fragment (Residues 855–1296) of the Toxin.

Minako Oshima, Makoto Hayakari, John L. Middlebrook and M. Zouhair Atassi

Mol. Immunol. (1997), submitted.

Antibodies and T cells against synthetic peptides of the C-terminal domain (H_C) of botulinum neurotoxin type A and their cross-reaction with H_C.

Minako Oshima, John L. Middlebrook, and M. Zouhair Atassi

Immunol. Letters (1997), Submitted.

Table I. Summary of Peptides Recognized by Horse Abs Against BoNT/A and Human and Mouse Abs Against Pentavalent Toxoid[a]

Peptide number	Sequence position	Horse	Human	Mouse
1	855–873	+++	−	±
2	869–887	−	+++	+++
3	883–901	+	−	+
4	897–915	±	−	−
5	911–929	+	++	−
6	925–943	−	+++	−
7	939–957	++	+	+
8	953–971	−	−	−
9	967–985	−	+	±
10	981–999	±	+++	−
11	995–1013	+	+++++	+
12	1009–1027	−	−	−
13	1023–1041	+	−	−
14	1037–1055	+	−	−
15	1051–1069	±	+++++	++
16	1065–1083	−	−	−
17	1079–1097	+	−	−
18	1093–1111	+	−	+
19	1107–1125	−	±	−
20	1121–1139	+	+	±
21	1135–1153	±	++	±
22	1149–1167	+	−	−
23	1163–1181	−	±	−
24	1177–1195	−	+++	++
25	1191–1209	++	±	−
26	1205–1223	+	−	−
27	1219–1237	−	+	−
28	1233–1251	−	+	−
29	1247–1265	±	++	−
30	1261–1279	++	+	−
31	1275–1296	+++	++	++

[a] For the purpose of this table, (+) or (−) assignments were based on net cpm values which, for human and mouse, were derived from the dilution that gave the highest binding. The symbols denote the following: (−), <1500 cpm; (±), 1500–3000 cpm; (+), 3000–7000 cpm; (++), 7000–15,000 cpm; (+++), 15,000–25,000 cpm; (++++), 25,000–35,000 cpm; (+++++), >35,000 cpm.

Table 2. The regions on the H_c domain of BoNT/A that are recognized by Abs and/or T cells after immunization of Balb/c and SJL mouse strains with pentavalent toxoid[a]

		Balb/c ($H\text{-}2^d$)		SJL ($H\text{-}2^s$)	
Peptide	Residue Numbers	Ab	T cells	Ab	T cells
1	855-873	-	-	+	-
2	869-887	++	-	+++	-
3	883-901	++	-	++	-
4	897-915	-	++	-	++++
5	911-929	-	-	±	+
6	925-943	+	-	+	+
7	939-957	+	++	+	+++
8	953-971	-	-	-	++
9	967-985	+	-	+	-
10	981-999	+	-	+	-
11	995-1013	+	-	+++	-
12	1009-1027	-	+	-	+
13	1023-1041	±	-	-	++
14	1037-1055	-	-	-	+
15	1051-1069	+	-	++	+++
16	1065-1083	-	-	-	+
17	1079-1097	-	-	-	++
18	1093-1111	-	-	+	+
19	1107-1125	±	-	+	+
20	1121-1139	-	-	+	++
21	1135-1153	++	-	±	+
22	1149-1167	-	-	±	+
23	1163-1181	-	-	-	++
24	1177-1195	+++	-	+++	+
25	1191-1209	-	-	±	+
26	1205-1223	-	-	±	-
27	1219-1237	-	-	-	-
28	1233-1251	-	-	+	+
29	1247-1265	-	-	-	+
30	1261-1279	-	-	+	-
31	1275-1296	++	-	++	++

Table 3 The proliferative response to the synthetic BoNT/A peptides of LNC obtained from BALB/c and SJL mouse strains after immunization with pentavalent toxoid

Peptide	Residue Numbers	Balb/c ($H\text{-}2^d$)		SJL ($H\text{-}2^s$)	
		Group 1 1 injection SI ± SD	Group 2 3 injections SI ± SD	Group 1 1 injection SI ± SD	Group 2 3 injections SI ± SD
1	855-873	0.94 ± 0.03	1.50 ± 0.13	0.94 ± 0.01	1.00 ± 0.02
2	869-887	0.96 ± 0.03	1.40 ± 0.29	0.69 ± 0.22	0.92 ± 0.07
3	883-901	0.98 ± 0.04	1.90 ± 0.25	0.58 ± 0.07	1.07 ± 0.15
4	897-915	**4.42 ± 0.13**[a]	**2.23 ± 0.11**	**12.47 ± 0.10**	**23.15 ± 1.61**
5	911-929	0.90 ± 0.38	1.40 ± 0.00	**3.49 ± 0.07**	2.08 ± 0.01
6	925-943	1.31 ± 0.01	**2.40 ± 0.03**	**3.20 ± 0.07**	**5.00 ± 0.69**
7	939-957	**4.35 ± 0.01**	**3.10 ± 0.23**	**5.30 ± 0.19**	**4.73 ± 0.05**
8	953-971	1.82 ± 0.01	**2.40 ± 0.06**	**4.35 ± 0.11**	**2.67 ± 0.43**
9	967-985	1.92 ± 0.07	1.30 ± 0.02	1.29 ± 0.20	**2.52 ± 0.15**
10	981-999	0.93 ± 0.01	1.30 ± 0.23	1.20 ± 0.16	0.95 ± 0.01
11	995-1013	0.95 ± 0.02	1.80 ± 0.10	1.14 ± 0.03	0.89 ± 0.05
12	1009-1027	**2.29 ± 0.11**	1.60 ± 0.20	**3.03 ± 0.20**	0.97 ± 0.18
13	1023-1041	1.09 ± 0.02	1.50 ± 0.01	**3.81 ± 0.27**	**2.65 ± 0.03**
14	1037-1055	0.82 ± 0.02	1.90 ± 0.29	**2.76 ± 0.03**	1.13 ± 0.13
15	1051-1069	0.95 ± 0.03	1.50 ± 0.13	**6.05 ± 0.27**	**9.81 ± 0.52**
16	1065-1083	1.03 + 0.05	1.50 ± 0.24	**2.88 ± 0.02**	**2.25 ± 0.04**
17	1079-1097	1.08 ± 0.01	1.10 ± 0.06	**3.76 ± 0.17**	1.23 ± 0.14
18	1093-1111	1.30 ± 0.12	1.30 ± 0.10	**2.94 ± 0.05**	**2.97 ± 0.96**
19	1107-1125	0.98 ± 0.11	1.70 ± 0.09	**2.92 ± 0.04**	2.07 ± 0.12
20	1121-1139	1.53 ± 0.05	1.50 ± 0.00	**4.13 ± 0.01**	**4.00 ± 0.03**
21	1135-1153	1.40 ± 0.00	1.50 ± 0.17	2.13 ± 0.08	**3.20 ± 0.03**
22	1149-1167	1.25 ± 0.03	**3.10 ± 0.11**	**3.10 ± 0.01**	1.41 ± 0.13
23	1163-1181	1.29 ± 0.07	1.50 ± 0.25	**3.80 ± 0.29**	1.17 ± 0.09
24	1177-1195	1.17 ± 0.03	1.30 ± 0.16	**2.77 ± 0.02**	1.21 ± 0.22
25	1191-1209	1.34 ± 0.07	1.70 ± 0.19	**3.26 ± 0.14**	1.48 ± 0.47
26	1205-1223	1.18 ± 0.00	1.20 ± 0.14	1.77 ± 0.14	1.09 ± 0.38
27	1219-1237	0.86 ± 0.11	1.50 ± 0.09	1.44 ± 0.04	**2.39 ± 0.06**
28	1233-1251	0.93 ± 0.17	0.90 ± 0.22	**2.65 ± 0.03**	0.65 ± 0.08
29	1247-1265	1.71 ± 0.05	1.30 ± 0.03	2.13 ± 0.13	**2.76 ± 0.20**
30	1261-1279	1.69 ± 0.07	**7.10 ± 0.43**	1.70 ± 0.14	**3.91 ± 0.16**
31	1275-1296	1.36 ± 0.03	1.30 ± 0.06	**3.85 ± 0.13**	**2.95 ± 0.00**
Controls					
Unrelated peptide		1.06 ± 0.09	0.98 ± 0.02	0.68 ± 0.25	0.74 ± 0.07
Myoglobin		0.98 ± 0.02	0.97 ± 0.04	1.17 ± 0.02	0.84 ± 0.09
Con A		**20.64 ± 0.65**	**30.00 ± 0.97**	**28.50 ± 0.30**	**36.90 ± 1.37**

[a]Boldface, hatched values are significantly higher than unstimulated controls (SI>2.0).

Table 4. Summary of peptides recognized by Abs and by T lymphocytes when H_C is used as immunogens in SJL and BALB/c mouse strains[a]

Peptide recognized		SJL (H-2^s)		BALB/c (H-2^d)	
Pept. No.	Sequence Position	Ab	T Cells	Ab	T Cells
1	855–873	+	–	+	–
2	869–887	++	–	++++	–
3	883–901	++	–	+++	–
4	897–915	+++	++++	–	–
5	911–929	–	+++	–	–
6	925–943	±	+++	+	–
7	939–957	+++	++++	+	+
8	953–971	–	++++	–	±
9	967–985	–	+++	++	–
10	981–999	+	–	+++	–
11	995–1013	+++	+	+	–
12	1009–1027	–	±	–	+
13	1023–1041	–	–	–	±
14	1037–1055	–	+	–	–
15	1051–1069	++	++	+	–
16	1065–1083	–	±	–	–
17	1079–1097	–	–	++++	–
18	1093–1111	+	–	++++	–
19	1107–1125	+	±	±	±
20	1121–1139	–	+	–	–
21	1135–1153	–	–	++	++
22	1149–1167	–	±	–	–
23	1163–1181	–	–	–	–
24	1177–1195	+++	–	++++	–
25	1191–1209	–	–	–	–
26	1205–1223	–	–	–	–
27	1219–1237	–	–	–	–
28	1233–1251	–	–	–	–
29	1247–1265	–	–	–	–
30	1261–1279	–	–	–	–
31	1275–1296	++++	–	++++	–

Table 5. Summary of reaction with immunizing peptide and H_C of immune responses elicited when individual BoNT/A peptides including T and/or Ab epitope are used as immunogens in SJL and BALB/c mouse strains[1]

Peptide immunogen		SJL ($H\text{-}2^s$)					BALB/c ($H\text{-}2^d$)				
Pept. No.	Sequence Position	Epitope for[2]	Ab pept.	Ab H_C	T Cell pept.	T Cell H_C	Epitope for	Ab pept.	Ab H_C	T Cell pept.	T Cell H_C
2	869–887	Ab	±	–	–	–	Ab	++++	++++	+++	–
3	883–901	Ab	–	–	±	–	Ab	++	++++	±	–
4	897–915	T, Ab	+++++	+++++	+++++	++++	n/e[3]	n/e	n/e	n/e	n/e
5	911–929	T	++++	+	++++	++++	n/e	n/e	n/e	n/e	n/e
6	925–943	T	+++++	–	+++	+++	n/e	n/e	n/e	n/e	n/e
7	939–957	T, Ab	+++++	+	+++++	++++	T	+++++	++	+++	+++
8	953–971	T	+++	–	++++	++++	n/e	n/e	n/e	n/e	n/e
9	967–985	T	–	–	±	–	n/e	n/e	n/e	n/e	n/e
10	981–999	Ab	+++++	++	+++	+	Ab	++++	++++	–	–
11	995–1013	Ab	++	±	–	–	n/e	n/e	n/e	n/e	n/e
12	1009–1027	n/e	n/e	n/e	n/e	n/e	T	+++++	+++	+++	+
15	1051–1069	T, Ab	+	+	+++	±	n/e	n/e	n/e	n/e	n/e
17	1079–1097	n/e	n/e	n/e	n/e	n/e	Ab	+++	+++	±	+
18	1093–1111	n/e	n/e	n/e	n/e	n/e	Ab	++	++	–	–
21	1135–1153	n/e	n/e	n/e	n/e	n/e	T, Ab	+++	+++	+++	–
24	1177–1195	Ab	++	+	±	–	Ab	+++	+++	++	–
31	1275–1296	Ab	+	+	++	–	Ab	++++	++++	++	–

Footnote to Table 5

[1]Assignment of positive and negative responses for the purpose of this table was based on net cpm values for Ab study and of S.I. values for T-cell study. For Ab binding, the symbols denote the following values: (–), less than 2,000 cpm; (±), 2,000–5,000 cpm; (+), 5,000–12,000 cpm; (++), 12,000–22,000 cpm; (+++), 22,000–40,000 cpm; (++++), 40,000–60,000 cpm; (+++++), >60,000 cpm. For T cell recognition, the symbols denote the following: (–), S.I. value less than 2.0; (±), S.I. 2.0–2.9; (+), S.I. 3.0–5.0; (++), S.I. 5.1–10.0; (+++), S.I. 10.1–30.0; (++++), S.I. 30.1–60.0; (+++++), S.I. ≥ 60.1. All the anti-peptide antisera were unresponsive to protein and peptide controls used. The LNC of all experiments were unresponsive to unrelated proteins or peptide but responded appropriately to Con A and LPS.

[2]When H_C is used as the immunogen.

[3]n/e: indicates that the peptide is neither an Ab nor a T-cell epitope in this mouse strain when it is immunized with H_C and therefore the peptide was not used as an immunogen in this mouse strain.

Table 6. Summary of immune responses elicited when an equimolar mixture of peptides containing T and B cell epitopes was used as an immunogen in SJL and BALB/c[1]

Pept. No.	SJL ($H\text{-}2^s$) Ab	SJL ($H\text{-}2^s$) T Cell	BALB/c ($H\text{-}2^d$) Ab	BALB/c ($H\text{-}2^d$) T Cell
2	++	–	+++	±
3	+++	+++	+++	–
4	+++++	++++	n/e[2]	n/e
5	±	++	n/e	n/e
6	+	+++	n/e	n/e
7	+++	++++	+++	+++
8	+++	++	n/e	n/e
9	+++	+++	n/e	n/e
10	++++	+++	+++	–
11	+	–	n/e	n/e
12	n/e	n/e	–	+++
15	±	+++	n/e	n/e
17	n/e	n/e	–	–
18	n/e	n/e	++	–
21	n/e	n/e	–	++
24	++	++	+++	±
31	+++	+++	++++	–
H_C	+++++	+++++	+++++	+++

[1,2] See footnote for Table 5.

Peptide Number	Sequence Position	Structure
1	855–873	K Y V D N Q R L L S T F T E **Y I K N I**
2	869–887	**Y I K N I** I N T S I L N L R **Y E S N H**
3	883–901	**Y E S N H** L I D L S R Y A S **K I N I G**
4	897–915	**K I N I G** S K V N F D P I D **K N Q I Q**
5	911–929	**K N Q I Q** L F N L E S S K I **E V I L K**
6	925–943	**E V I L K** N A I V Y N S M Y **E N F S T**
7	939–957	**E N F S T** S F W I R I P K Y **F N S I S**
8	953–971	**F N S I S** L N N E Y T I I N **C M E N N**
9	967–985	**C M E N N** S G W K V S L N Y **G E I I W**
10	981–999	**G E I I W** T L Q D T Q E I K **Q R V V F**
11	995–1013	**Q R V V F** K Y S Q M I N I S **D Y I N R**
12	1009–1027	**D Y I N R** W I F V T I T N N **R L N N S**
13	1023–1041	**R L N N S** K I Y I N G R L I **D Q K P I**
14	1037–1055	**D Q K P I** S N L G N I H A S **N N I M F**
15	1051–1069	**N N I M F** K L D G C R D T H **R Y I W I**
16	1065–1083	**R Y I W I** K Y F N L F D K E **L N E K E**
17	1079–1097	**L N E K E** I K D L Y D N Q S **N S G I L**
18	1093–1111	**N S G I L** K D F W G D Y L Q **Y D K P Y**
19	1107–1125	**Y D K P Y** Y M L N L Y D P N **K Y V D V**
20	1121–1139	**K Y V D V** N N V G I R G Y M **Y L K G P**
21	1135–1153	**Y L K G P** R G S V M T T N I **Y L N S S**
22	1149–1167	**Y L N S S** L Y R G T K F I I **K K Y A S**
23	1163–1181	**K K Y A S** G N K D N I V R N **N D R V Y**
24	1177–1195	**N D R V Y** I N V V V K N K E **Y R L A T**
25	1191–1209	**Y R L A T** N A S Q A G V E K **I L S A L**
26	1205–1223	**I L S A L** E I P D V G N L S **Q V V V M**
27	1219–1237	**Q V V V M** K S K N D Q G I T **N K C K M**
28	1233–1251	**N K C K M** N L Q D N N G N D **I G F I G**
29	1247–1265	**I G F I G** F H Q F N N I A K **L V A S N**
30	1261–1279	**L V A S N** W Y N R Q I E R S **S R T L G**
31	1275–1296	**S R T L G** C S W E F I P V D D G W G E R P L

Fig. 1. Synthetic overlapping peptides of the protective H_C region of BoNT/A. The 31 peptides shown started at residue 855 and covered the entire sequence of H_C (residues 860–1296 of the H chain). Each peptide overlapped by 5 residues with each of its adjacent neighbors and the regions of overlap are shown in bold type.

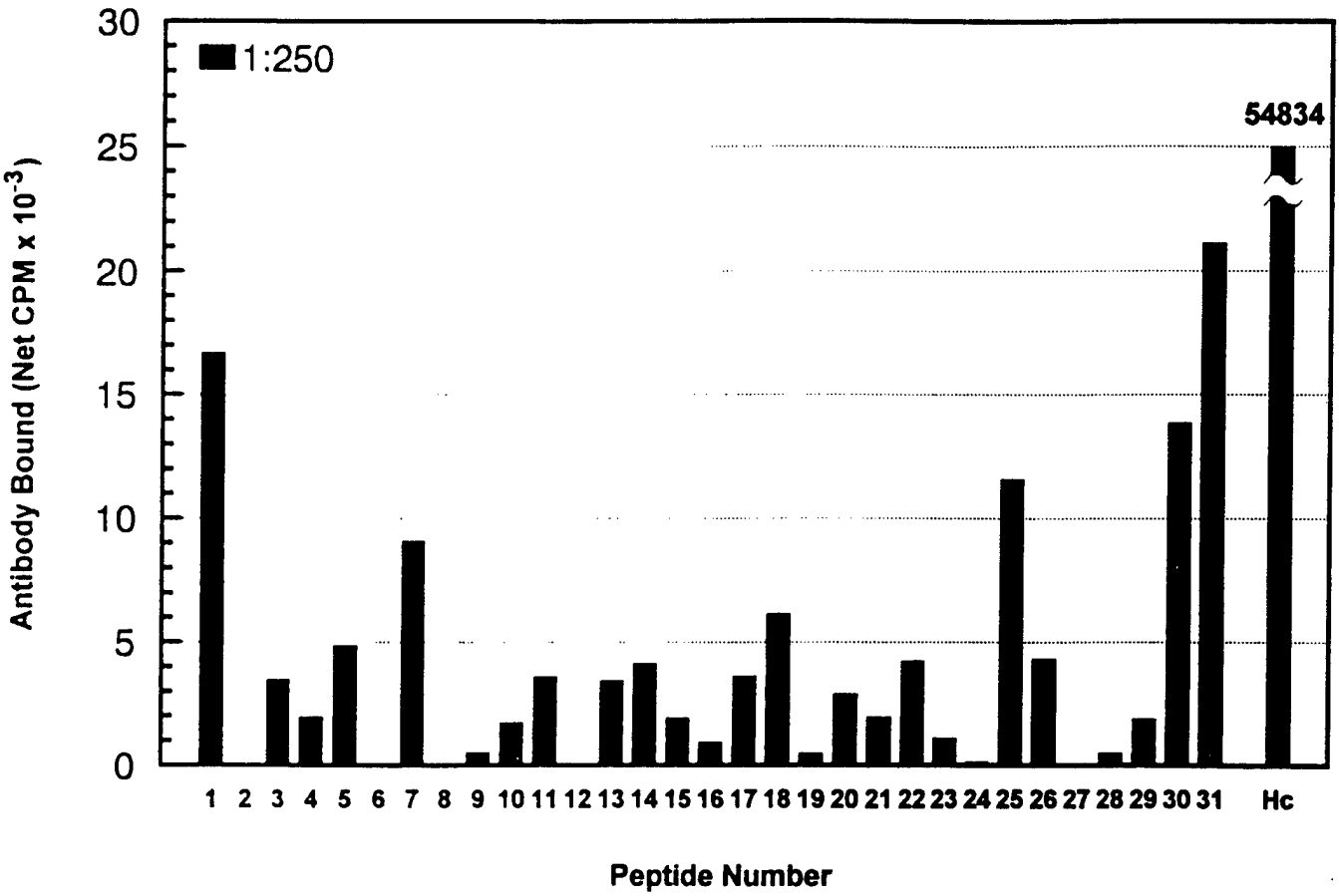

Fig. 2. Binding of horse anti-BoNT/A antibodies to the overlapping BoNT/A peptides and to H_C. Binding was determined by solid-phase plate RIA using the antiserum at a dilution of 1:250 (vol/vol). The results were corrected for nonspecific binding of the antibodies to unrelated protein (BSA) and of preimmune sera to the peptides and to H_C. The data are expressed in net cpm and represent the average of triplicate analyses which varied ±2.0% or less. For details, see the text.

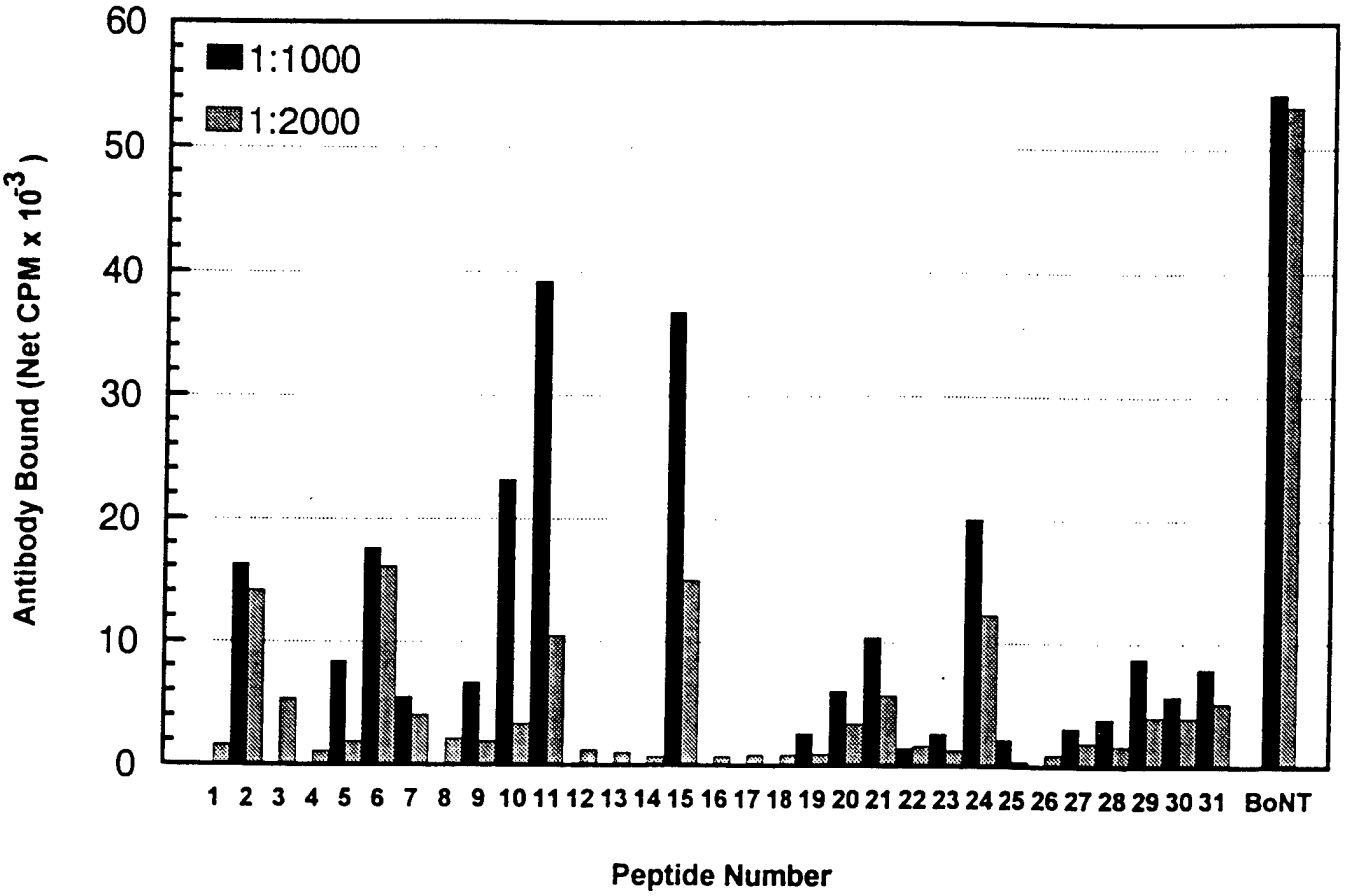

Fig. 3. Binding of human anti-toxoid antibodies to toxoid and to the overlapping peptides of the H_C domain of BoNT/A. Binding was done by solid-phase plate RIA at dilutions of 1:1000 and 1:2000 (vol/vol) of a 105 mg/ml solution of the IgG fraction of the antibody and has been corrected for nonspecific binding of the antibodies to an unrelated protein (BSA) and of nonimmune human IgG to the peptides and to BoNT/A. The results are given in net cpm of bound antibody and represent the average of triplicate analyses which varied ±2.0% or less. For details see the text.

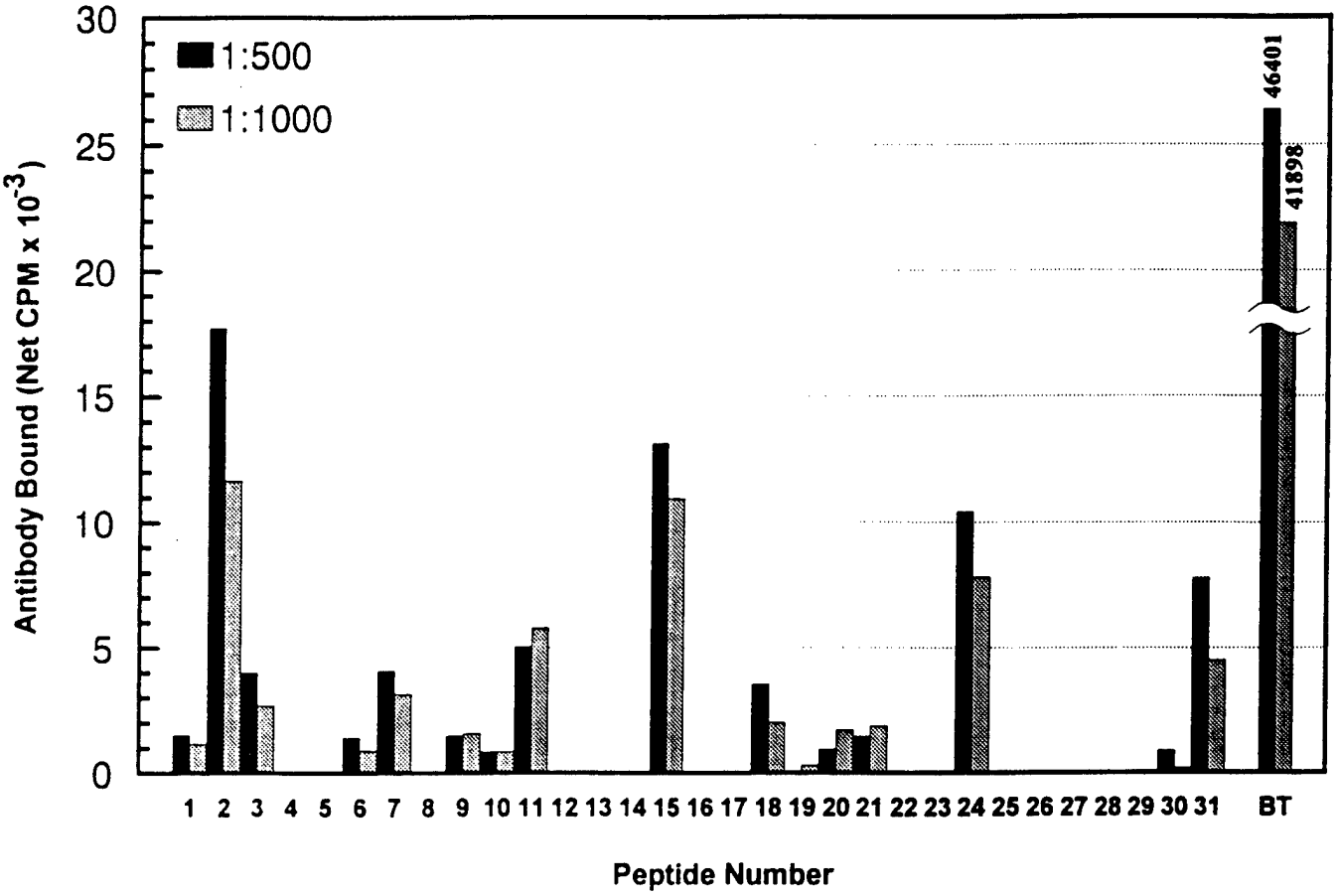

Fig. 4. Binding of outbred (ICR) mouse antisera to the overlapping synthetic H_C peptides of BoNT/A. Binding was determined at two dilutions (1:500 and 1:1000, vol/vol) of the antisera and the results, which are expressed in net cpm, have been corrected for nonspecific binding of the antisera to unrelated protein (BSA) and of the preimmune sera to the toxoid and to each of the synthetic peptides. For details, see the text.

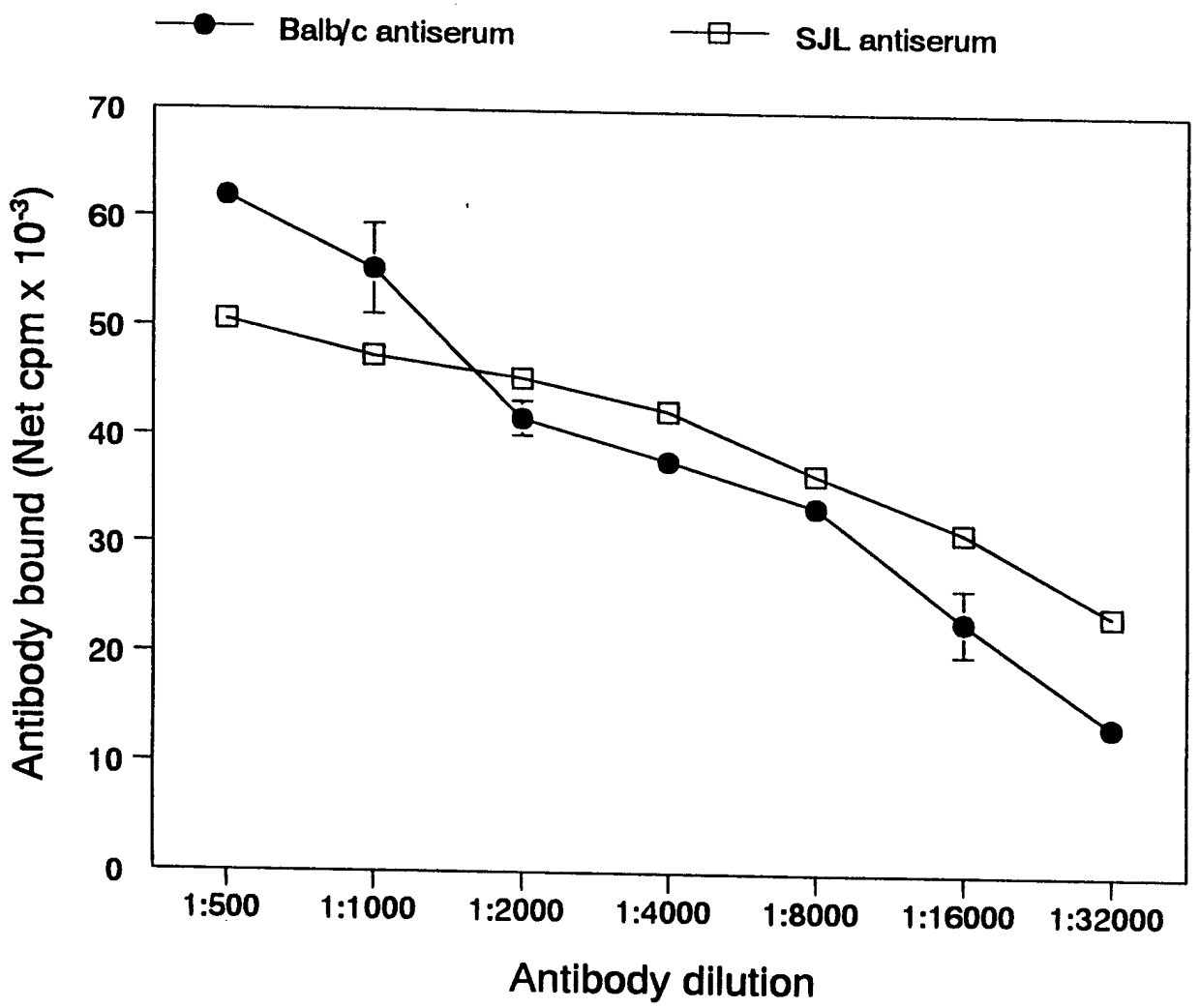

FIG. 5. Titration of Abs in anti-toxoid antisera from Balb/c and SJL mice. Specific Ab binding was determined by solid-phase RIA. The results are expressed in net cpm ± SD after correction for non-specific binding of Abs to unrelated protein (BSA) and of preimmune sera to toxoid.

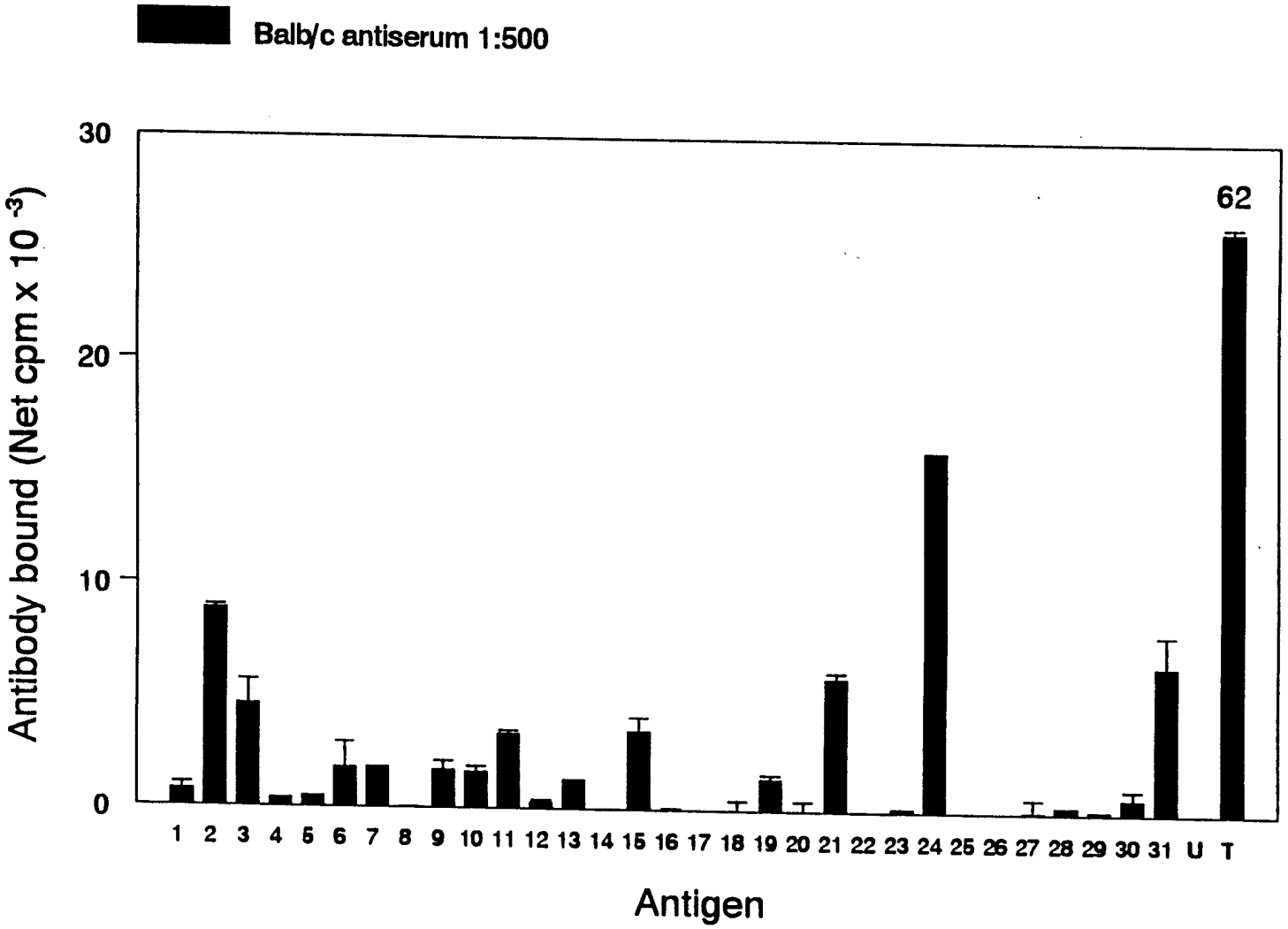

FIG. 6. Binding of Balb/c anti-toxoid Abs to toxoid (T), to the synthetic overlapping peptides of BoNT/A and to the unrelated synthetic peptide (U) used as a negative control. For RIA, the antiserum was diluted 1:500 (v/v). Results are given in net cpm $\pm$ SD of triplicate analyses and have been corrected for nonspecific binding of the Abs to unrelated protein (BSA) and of the preimmune sera to the toxoid and to each of the synthetic peptides. The value on the top of bar designated T shows the amount of Abs (61,957 $\pm$ 778 net cpm) bound to the toxoid.

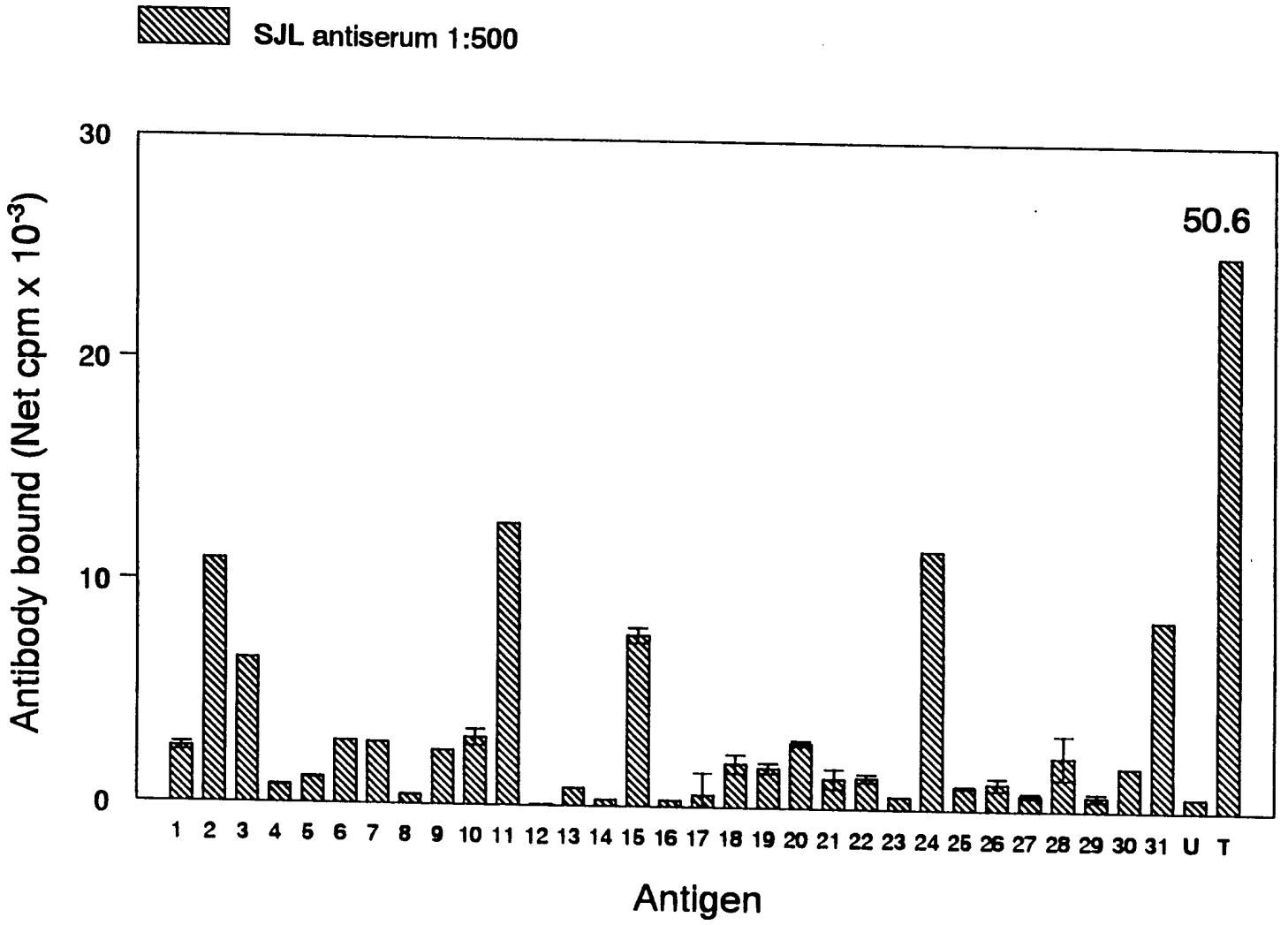

FIG. 7. Binding of SJL anti-toxoid Abs to toxoid (T), to the synthetic overlapping peptides (1–31) of BoNT/A and to the unrelated peptide (U). Data are expressed and corrected as in Figure 6. Binding of antibodies to toxoid gave 50,575 ± 57 cpm as indicated on top of the bar T.

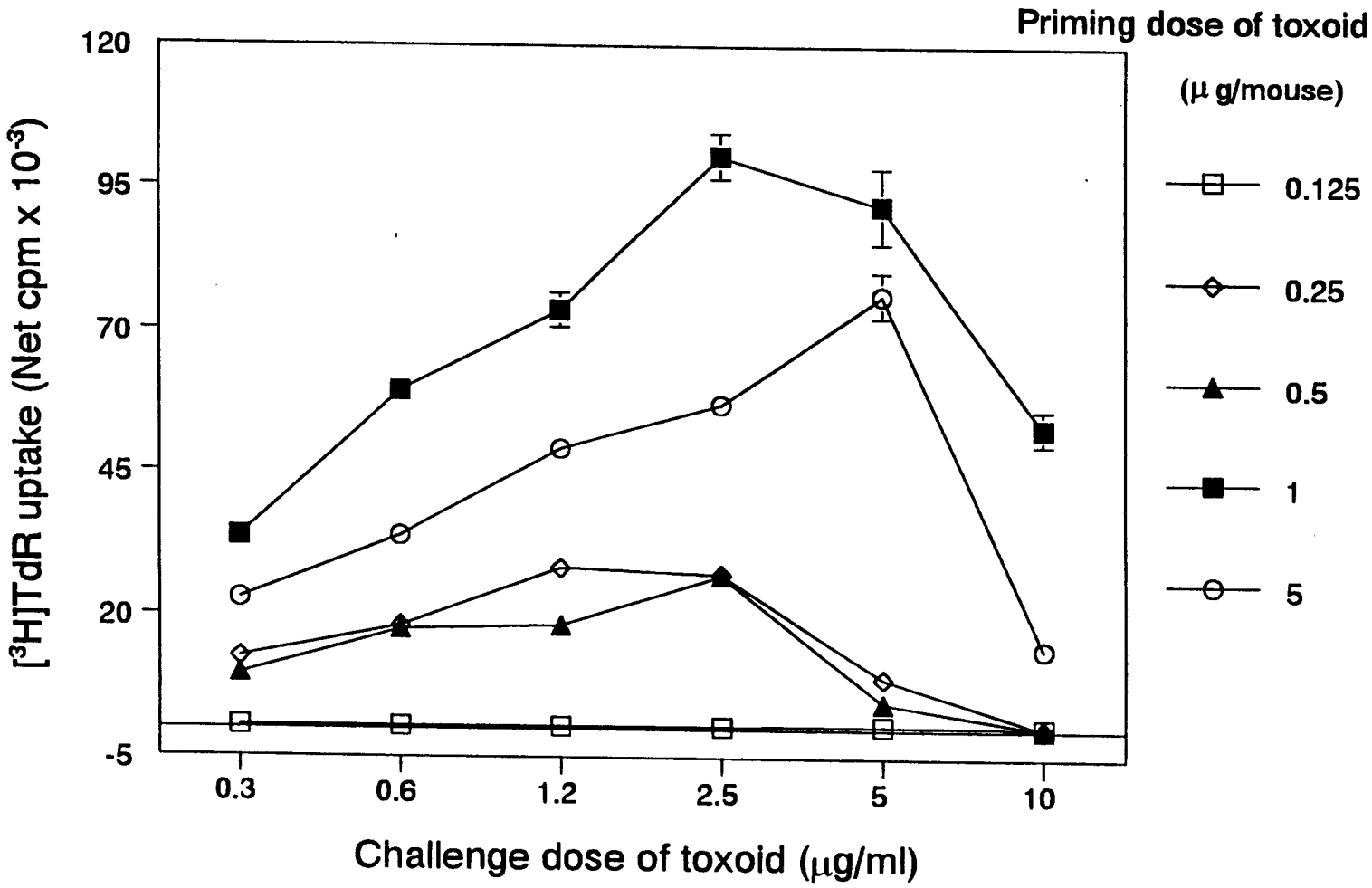

FIG. 8. Dependence on the *in vitro* toxoid challenge dose of the proliferative response of LNC from Balb/c mice that were primed with different doses (0.125–5 μg/mouse) of pentavalent toxoid (BoNTs A, B, C, D, E). Experimental details are described in Material and Methods.

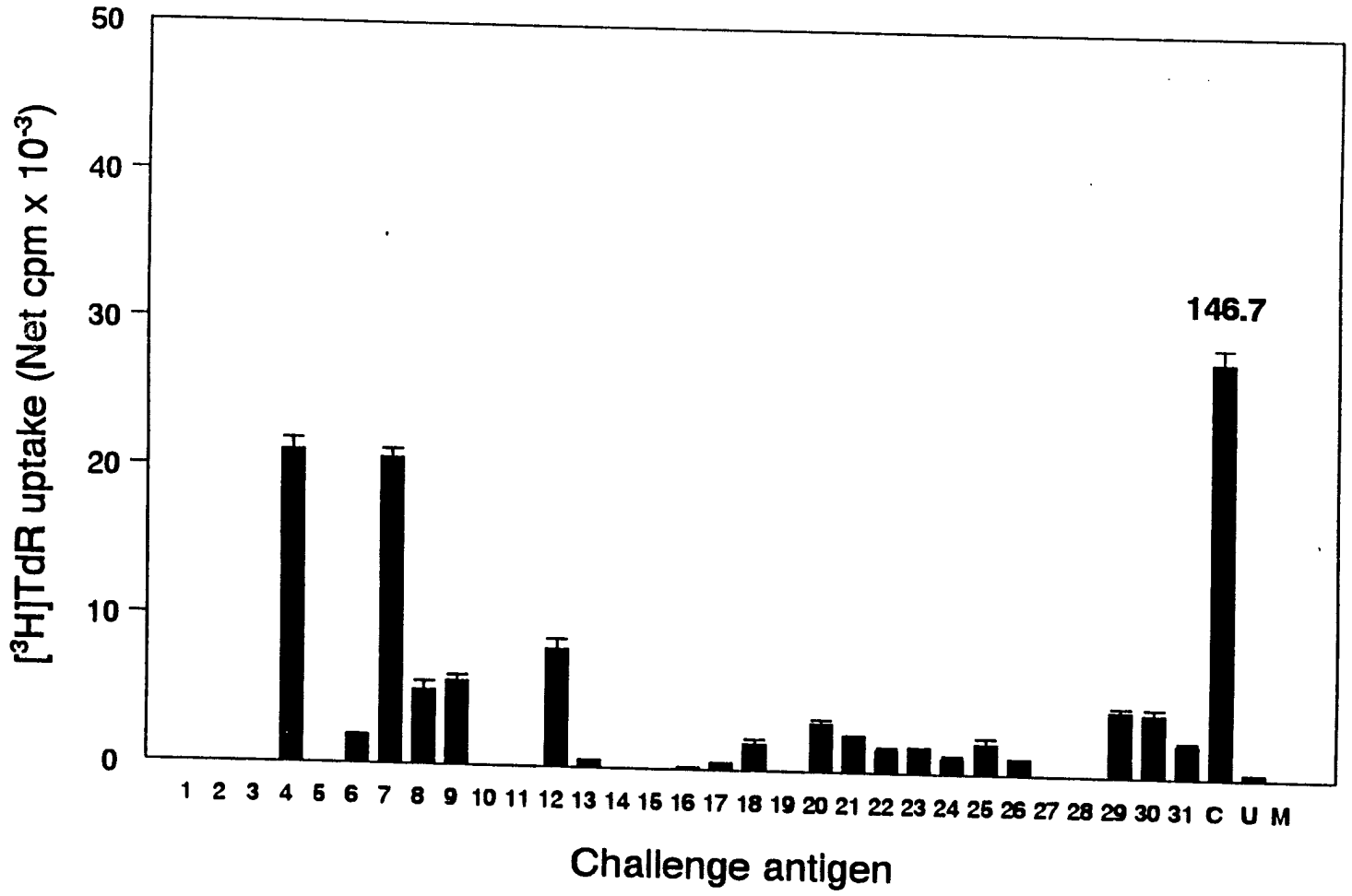

FIG. 9. Proliferative response of LNC from toxoid-primed Balb/c mice to *in vitro* challenge with the synthetic overlapping BoNT/A peptides. Numbers 1–31 under the abscissa refer to the peptide numbers shown in Figure 1. Controls included H_C (C), unrelated synthetic peptide (U) and myoglobin (M). Results are expressed in net cpm $\pm$ SD of triplicate cultures at the optimal stimulation dose of each challenge antigen. The value on the top of the bar marked C indicates the vigorous T cell response to H_C (146,684 $\pm$ 1,801 cpm). The amount of [H^3]TdR incorporated by unstimulated cells was 6,166 $\pm$ 53 cpm.

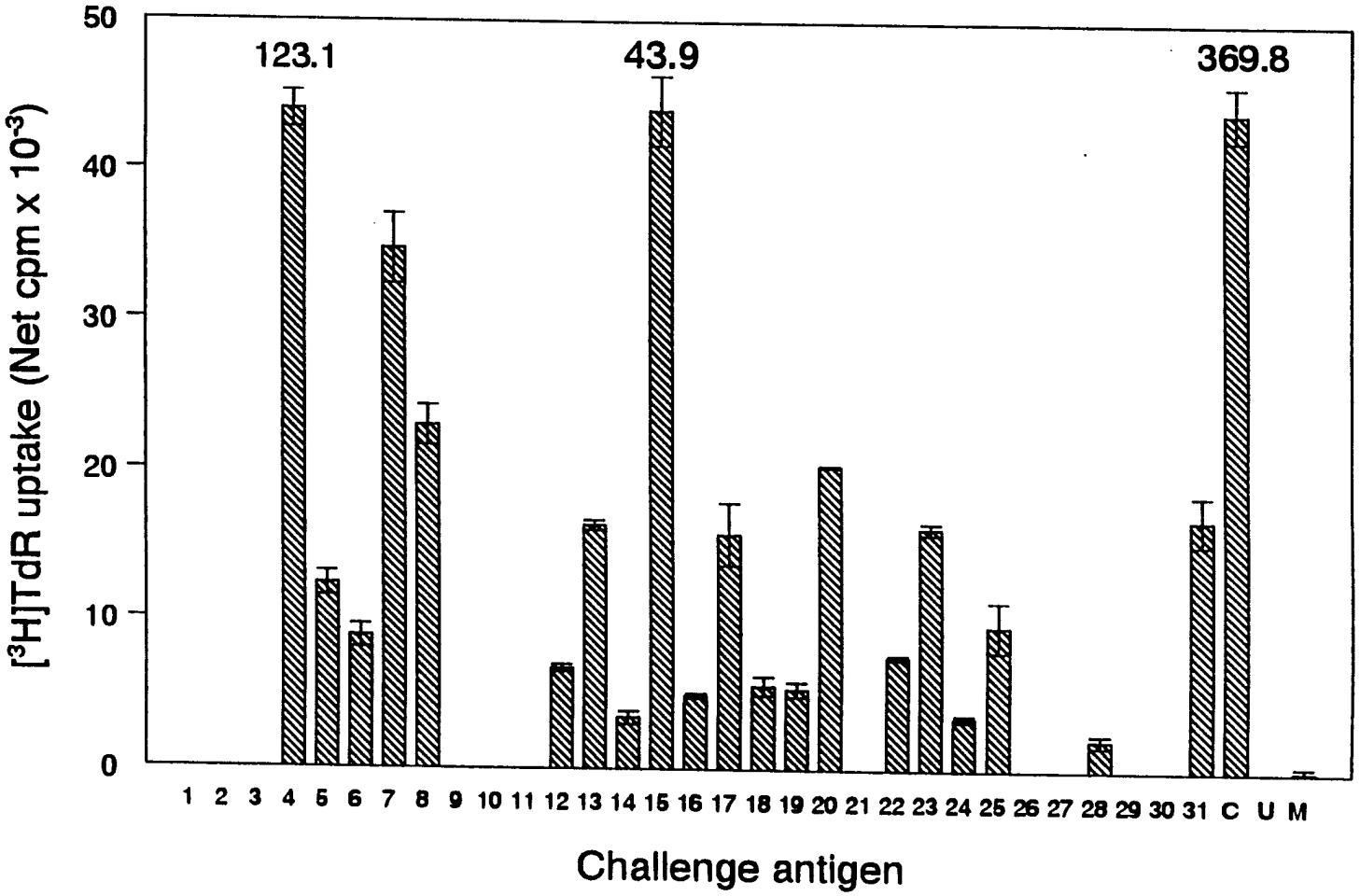

FIG. 10. Proliferative response of LNC from toxoid-primed SJL mice to *in vitro* challenge with the synthetic overlapping peptides of BoNT/A. Numbers and symbols of the antigens are as in Figure 3. The values on top of the bars show the strong response of T cells to the challenge with peptide 4 (123,120 ± 1,219 cpm), peptide 15 (43,912 ± 3,335 cpm) and H_C (369,801 ± 1,800 cpm). The level of [H^3]TdR incorporation in the absence of any antigenic stimulus was 12,331 ± 97 cpm.

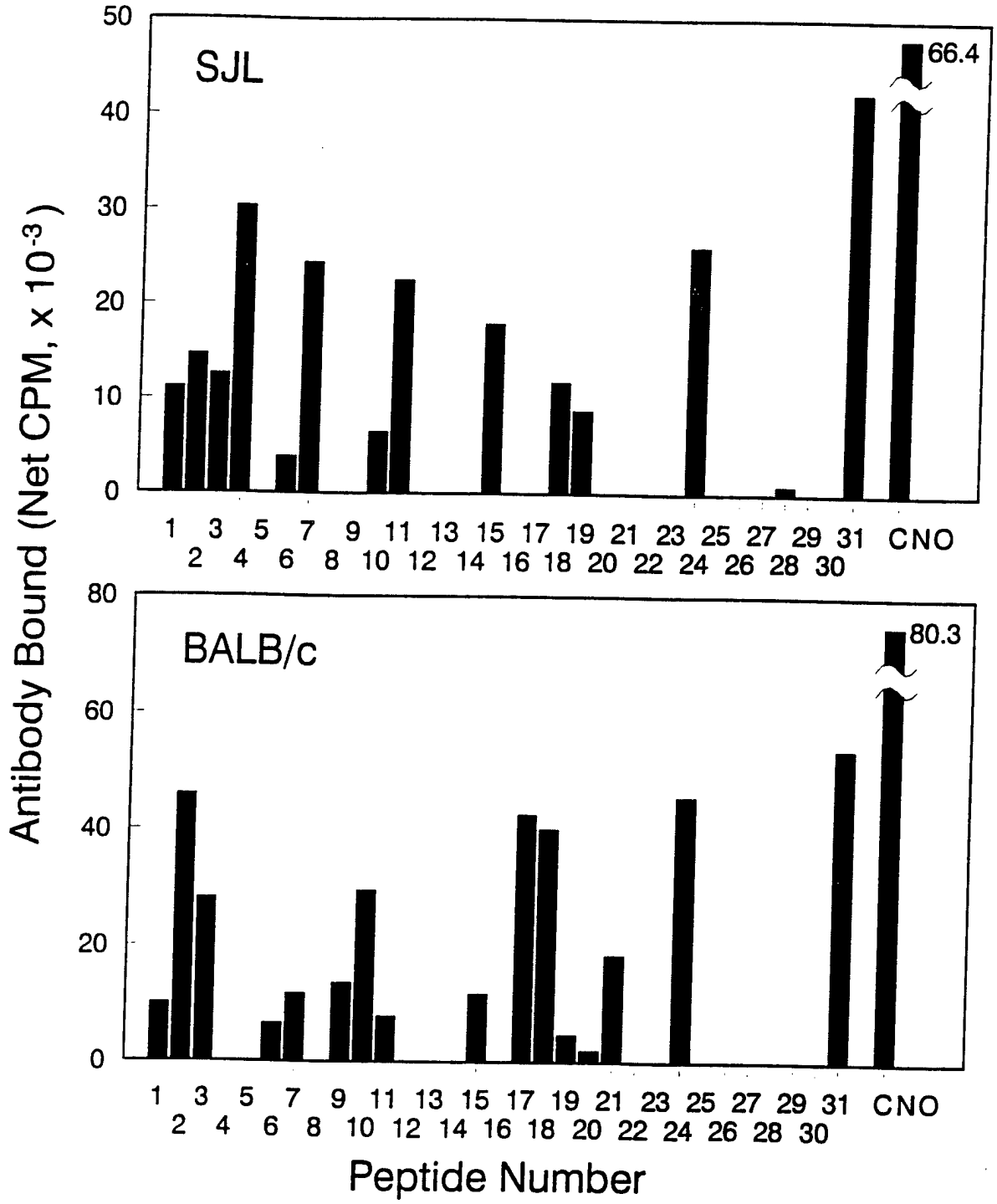

FIG. 11. Ab binding to the synthetic BoNT/A peptides of antisera from SJL and BALB/c after four immunizations (12 weeks after initial injection) with H_C (SJL, 0.5 g/mouse; BALB/c, 0.25 μg/mouse). The diagram shows the net cpm in which the average binding value of the same antigen to the SJL preimmune sera was subtracted. Numbers 1–31 refer to the peptides shown in Fig. 1. Additional antigen letter symbols are: C, H_C; N, unrelated synthetic peptide; O, ovalbumin.

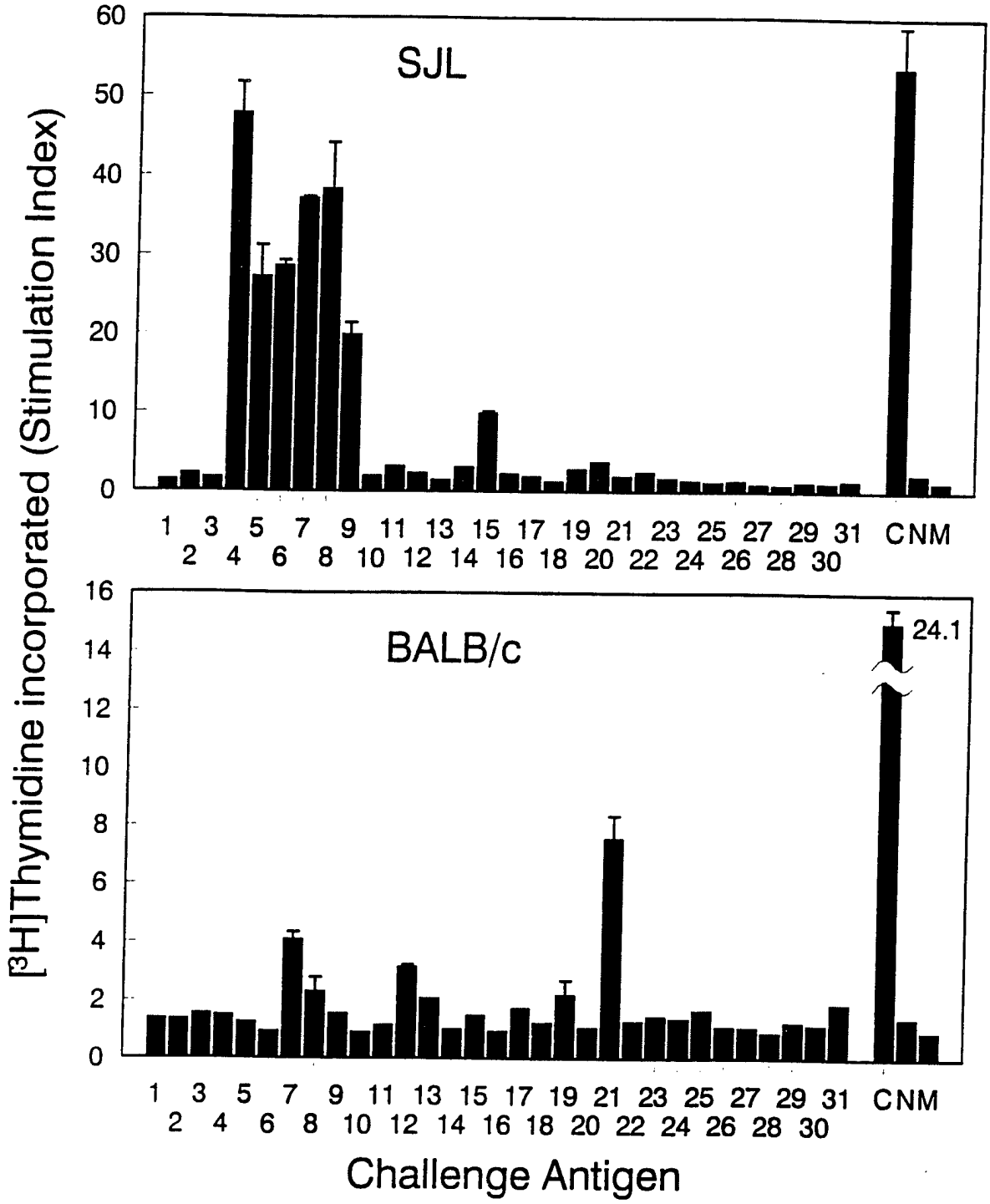

FIG. 12. Presentation showing the *in vitro* proliferative response to the BoNT/A peptides 1–31 of LNC from SJL and BALB/c mice primed with 0.25 g/mouse of H_C of BoNT/A. The diagram shows the S.I. at the optimum challenge doses of each peptide and H_C. Unstimulated cells gave 2330 ± 168 cpm and 3534 ± 141 for BALB/c. Numbers 1–31 refer to the peptides shown in Fig. 1. Other antigen letter symbols are: C, H_C; N, unrelated synthetic peptide; M, myoglobin.

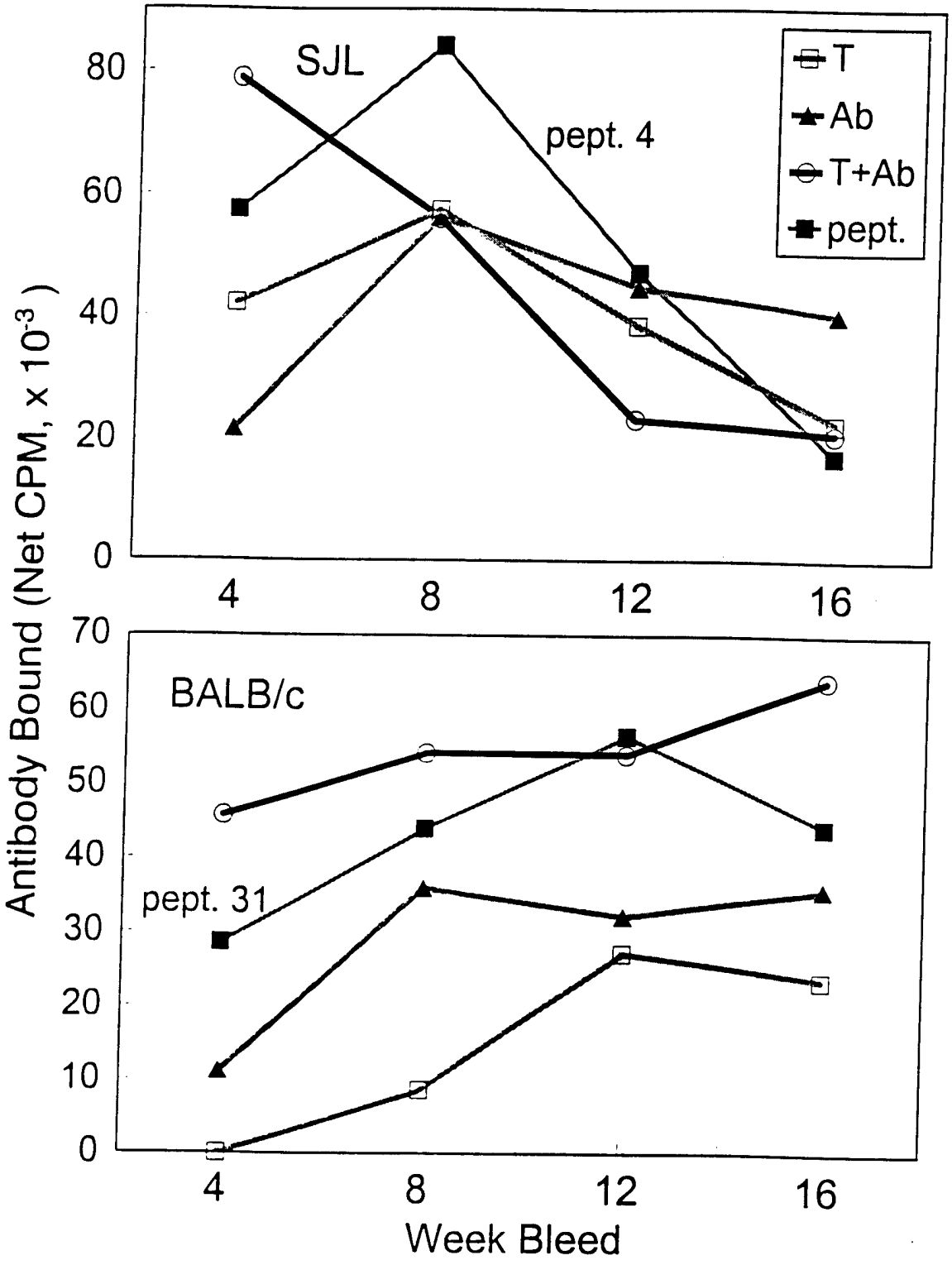

FIG. 13. Binding to Hc of Abs against three peptide mixtures or against peptide 4 (SJL) and peptide 31 (BALB/c) obtained at 4, 8, 12 and 16 weeks. SJL and BALB/c mice were immunized with an equimolar mixture of peptides containing T and/or Ab epitopes (when Hc is the immunogen [8]) or with individual peptides at 0, 3, 7, 11 and 15 weeks. Preimmune sera were used as negative controls, and their values were subtracted to obtain the net cpm. For details see text.

Sequence position	Toxin type	Structure
869–887 (peptide 2)	A	YIKNIIINTSILNLRYESNH
	B	YNSEILNNIILNLRYKDNN
	C	YFNNINDSKILSLQNRKNT
	D	YFNSINDSKILSLQNKKNA
	E	FFKRIKSSSVLNMRYKNDK
	F	LYKKIKDSSILDMRYENNK
	G	YISNISSNAILSLSYRGGR
	Te	IDVILKKSTILNLDINNDI
883–901 (peptide 3)	A	YESNHLIDLSRYASKINIG
	B	YKDNNLIDLSGYGAKVEVY
	C	NRKNTLVDTSGYNAEVSEE
	D	NKKNALVDTSGYNAEVRVG
	E	YKNDKYVDTSGYDSNININ
	F	YENNKFIDISGYGSNISIN
	G	YRGGRLIDSSGYGATMNVG
	Te	INNDIISDISGFNSSVITY
925–943 (peptide 6)	A	EVILKNAIVYNSMYENFST
	B	RVTQNQNIIFNSVFLDFSV
	C	IVTQNENIVYNSMYESFSI
	D	IVNLNNNILYSAIYENSSV
	E	NISQNDYIIYDNKYKNFSI
	F	NIAQNNDIIYNSRYQNFSI
	G	TAHQSKFVVYDSMFDNFSI
	Te	IVHKAMDIEYNDMFNNFTV
939–57 (peptide 7)	A	ENFSTSFWIRIPKYFNSIS
	B	LDFSVSFWIRIPNIRMMVY
	C	ESFSISFWIRINK-WVSNL
	D	ENSSVSFWIKISKDLTNSH
	E	KNFSISFWVRIPNYDNKIV
	F	QNFSISFWVRIPKHYKPMN
	G	DNFSINFWVRTPKYNNNDI
	Te	NNFTVSFWLRVPKVSASHL
953–971 (peptide 8)	A	FNSISL---NNEYTIINCM-ENN
	B	RMMVYKIIFIMNIQIINCM-KNN
	C	WVSNLP-----GYTIIDSV-KNN
	D	LTNSH-----NEYTIINSI-EQN
	E	DNKIVNV--NNEYTIINCMRDNN
	F	YKPMNH---NREYTIINCMGNNN
	G	NNNDIQTYLQNEYTIISCI-KND
	Te	SASHLEQYGTNEYSIISSMKKHS
967–985 (peptide 9)	A	CM-ENN----SGWKVSLNYG---EIIW
	B	CM-KNN----SGWKISIRGN---RIIW
	C	SV-KNN----SGWSIGIISN---FLVF
	D	SI-EQN----SGWKLCIRNG---NIEW
	E	CMRDNN----SGWKVSLNHN---EIIW
	F	CMGNNN----SGWKISLRTVRDCEIIW
	G	CI-KND----SGWKVSIKGN---RIIW
	Te	SMKKHSLSIGSGWSVSLKGN---NLIW
981–999 (peptide 10)	A	G---EIIWTLQDTQEIKQRVVF
	B	N---RIIWTLIDINGKTKSVFF
	C	N---FLVFTLKQNEDSEQSINF
	D	G---NIEWILQDVNRKYKSLIF
	E	N---EIIWTLQDNAGINQKLAF
	F	VRDCEIIWTLQDTSGNKENLIF
	G	N---RIIWTLIDVNAKSKSIFF
	Te	N---NLIWTLKDSAGEVRQITF

Sequence position	Toxin type	Structure
995–1013 (peptide 11)	A	QRVVFKYSQMINISDYI-NR
	B	KSVFFEYNIREDISEYI-NR
	C	QSINFSYDISNNAPGY--NK
	D	KSLIFDYSESLSHTGYT-NK
	E	QKLAFNYGNANGISDYI-NK
	F	ENLIFRYEELNRISNYI-NK
	G	KSIFFEYSIKDNISDYI-NK
	Te	RQITFR-DLPDKFNAYLANK
1009–1027 (peptide 12)	A	DYI-NRWIFVTITNNRLNNS
	B	EYI-NRWFFVTITNN-LNKA
	C	GY--NKWFFVTVTNNMMGNM
	D	GYT-NKWFFVTITNNIMGYM
	E	DYI-NKWIFVTITNDRLGDS
	F	NYI-NKWIFVTITNNRLGNS
	G	DYI-NKWFSITITNDRLGNA
	Te	AYLANKWVFITITNDRLSSA
1051–1069 (peptide 15)	A	NNIMFKLDG--------CRDTHRYIWI
	B	GEIIFKLDGDIDR---------TQFIWM
	C	KTITFEINKIPDTGLITSDSDNINMWI
	D	KTIVFGIDENID--------ENQMIWI
	E	DNILFKIVN--------CSYT-RYIGI
	F	DNILFKIVG--------CDDE-TYVGI
	G	NDIDFKLIN--------CTDTTKFVWI
	Te	NNITLKLDR--------CNNNNQYVSI
1093–111 (peptide 18)	A	NSGILKDFWGDYLQYDKPY
	B	YSEYLKDFWGNPLMYNKEY
	C	YTNVVKDYWGNDLRYNKEY
	D	LRNVIKDYWGNPLKFDTEY
	E	NTNILKDFWGNYLLYDKEY
	F	DPSILKNYWGNYLLYNKKY
	G	STNTLKDFWGNPLRYDTQY
	Te	SITFLRDFWGNPLRYDTEY
1177–1195 (peptide 24)	A	NDRVYIN-VVVKNKEYRL-AT
	B	EDYIYLD-FFNLNQEWRV---
	C	GDILYFD-MTINNKAYNL-FM
	D	GDNIILH-MLYNSRKYMI-IR
	E	NDQVYINFVASKTHLFPL-YA
	F	NDLAYIN-VVDRGVEYRL-YA
	G	GDYIYLNIDNISDESYRV-YV
	Te	GDFIKLY-VSYNNNEHIVGYP
1275–1296 (peptide 31)	A	SRT-----LGCSWEFIPVDDGWGERPL
	B	PYNLK---LGCNWQFIPKDEGWTE
	C	NYASLLESTSTHWGFVPVSE
	D	NYETKLLSTSSFWKFISRDPGWVE
	E	TNS-----NGCFWNFISEEHGWQEK
	F	TSS-----NGCFWSSISKENGWKE
	G	KLR-----LGCNWQFIPVDEGWTE
	Te	I-------LGCDWYFVPTDEGWTND

Fig. 14

FIG. 14. Comparative alignment of BoNT types A through G and TeTX within 13 peptide regions on Hc. Bold letters in type A show the residues that are identical or similar in one or more of the toxin types listed. In types B through G and in TeTX (Te in the figure), residues identical to those of BoNT/A are in boldface type. Bold and italic letters represent the residues in which conservative replacements have occurred. Regions which have 5 or more continuous residues identical or similar to the corresponding BoNT/A sequence are underlined.